UNIVERSIDAD AUTÓNOMA DE MADRID
FACULTAD DE CIENCIAS
DEPARTAMENTO DE BIOLOGÍA

VARIACIÓN FENOTÍPICA Y MOVILIDAD: PAPEL EN LA COLONIZACIÓN DE LA RIZOSFERA POR *Pseudomonas fluorescens*

FRANCISCO MANUEL MARTÍNEZ GRANERO
Madrid, 2006

Memoria presentada para optar al Grado de
Doctor en Ciencias Biológicas

Fdo. Francisco Manuel Martínez Granero
Licenciado en Ciencias Químicas

Los directores

Fdo. Dra. Marta Martín Basanta
Profesor Contratado Doctor
Dpto. Biología
Universidad Autónoma de Madrid

Fdo. Rafael Rivilla Palma
Profesor Titular
Dpto. Biología
Universidad Autónoma de Madrid

AGRADECIMIENTOS

Agradezco enormemente la realización de este trabajo a mis directores Marta y Rafa, por encauzarme y aconsejarme en este arduo camino que es la ciencia, y como no por esos aperitivos en la cafetería y esas comidas estupendas de Navidad. Además, también me gustaría agradecerles la oportunidad que he tenido de ver sitios nuevos en cada congreso al que he ido.

También les doy las gracias a todos los profesores de Biología General, bueno Fisiología Vegetal. A mi paisana Paqui por esas tertulias a la hora de la comida. A Paco por esos momentos mañaneros en la piscina, cuando aún estábamos dormidos. A Arse y Marisa por preocuparse siempre por mi búsqueda de trabajo. A Javi por enseñarme que es fácil abstraerse, muy útil si te sientas cerca de María. A Elvira, por sus dotes de liderazgo en la cocina. A Pili, por esas risillas que se oían salir de su despacho cada vez que Dani hacía alguno de sus comentarios. Al resto de profesores: Eduardo Marco, Mabel, Flor, Eduardo Fernández, Eva, Mari Carmen, Antonio, Luís. Y como olvidar al Dr. Ildefonso Bonilla, gracias por acogerme dentro del grupo y enseñarme que la medicina alternativa funciona.

A mi gran familia, los becarios. A Esther, por esos “Nesteas y charletas” en la cafetería, ya se que el mundo de las cianobacterias existe. A David, por ser el gran padre de todos los becarios de la Unidad. A Miguelón, por sus inconmensurables ayudas con el frío mundo de la informática y su ayuda con los experimentos de última hora. A Dani y María, por hacerme más amena y entretenida la estancia en el laboratorio, todo hubiese sido más aburrido sin vuestro toque. A Vir, por ayudarme a controlar a María (no te enfades María, que en el fondo te quiero) y por el buen rollito que hay en la sala de becarios. A Roke, por enseñarme que la política es más entretenida que la ciencia. A las niñas (Ana y Emma), por ir siempre con una sonrisa, aunque hubiese que repetir los experimentos. A Silvia, “la funcionaria”, por ayudarme tanto con la realización de

esta tesis. Al resto de becarios: David Velázquez, Lars, Delia, Celia, Paloma, Nacho, Carolina,…

También me gustaría dar las gracias a aquellos que formaron parte de esta Unidad pero que ya no están: Isa, Dani, Yoanna, Nacho, Juan, Majo, María Sánchez (gracias por cuidarme en mi primer congreso internacional), etc.

A Amaya, por aguantarme tanto dentro como fuera del laboratorio. A Raquel y Pili, mis niñas de citometría, por estar siempre pendiente de mí en los buenos y en los malos momentos. A Kiki, por no pensar que los de ciencia somos bichos raros. A mi mami madrileña, por su cariño y esas estupendas comiditas que nos prepara. Al fondo cubano (Ailén, Leydis, etc), por preocuparse tanto, quererme y apoyarme.

A mi grupo de la carrera (Paloma, Edu, Rocío, Silvia, Enrique, Luís, Alicia y Javi), que se que están esperando impacientes el día de la tesis porque saben que habrá comilona. Y a mis amigos del pueblo (Gerardo, Carmen, José Julián, Luís, Sara y el nuevo matrimonio Montse-Alejandro), que se han preocupado tanto durante estos últimos meses de tesis.

A Fernando y Lali porque se que han estado muy pendientes de toda la trayectoria de estos años.

A mi Tati por estar siempre a mi lado, apoyándome y dándome ánimo para no desfallecer en el camino. Gracias por formar parte de mi vida.

A toda mi familia por comprenderme y apoyarme durante estos años. A mis tías/os por acogerme en su casa como si fuese la mía. Por último me gustaría dar las gracias especialmente a mis padres y mi hermana.

ÍNDICE

ÍNDICE

CAPÍTULO 1: Introducción general

CAPÍTULO 2: Analysis of *Pseudomonas fluorescens* F113 genes implicated in flagellar filament synthesis and their role in competitive root colonization

CAPÍTULO 1

Introducción general

1. PSEUDOMONAS

La familia *Pseudomonadaceae* fue establecida por Winslow y col. (1917) a principios del siglo XX, agrupando al género *Pseudomonas* y a un gran número de géneros dispares entre sí, muchos de los cuales se han ido reclasificando a lo largo de los años. La familia *Pseudomonadaceae* engloba microorganismos saprófitos de vida libre en suelos, ecosistemas acuáticos y otros hábitats, aunque también se pueden encontrar asociados a plantas y animales, en muchas ocasiones como agentes patógenos (Palleroni, 1992; Schroth *et al.*, 1992).

Las bacterias pertenecientes a esta familia se caracterizan por ser bacilos Gram negativos, rectos o ligeramente curvados, con un diámetro de 0.5-1 μm y una longitud de 1.5-5 μm. Son bacterias móviles debido a la presencia de uno o varios flagelos polares, aunque en algunas especies pueden aparecer flagelos laterales más pequeños. Son quimioorganotrofos y aeróbicos estrictos, sin embargo, en ciertos casos pueden usar el nitrato como aceptor de electrones permitiendo una cierta actividad metabólica en condiciones anaeróbicas, como *P. aeruginosa* que es capaz de crecer lentamente en anaerobiosis (Vander Wauven *et al.*, 1984).

La familia *Pseudomonadaceae*, que pertenece a la clase de las gammaproteobacterias, abarca los géneros *Pseudomonas, Cellvibrio* y *Azotobacter*. A su vez, el género *Pseudomonas*, que presenta un contenido en G+C de 59 a 68 mol%, se ha subdividido en varios subgrupos. El término pseudomonas (bacterias parecidas a *Pseudomonas*) se ha utilizado mucho para describir estirpes en las que la taxonomía no estaba muy bien establecida. Sin embargo en los últimos años, se ha hecho una distinción entre el género *Pseudomonas* en su sentido más estricto (γ–proteobacterias) y los géneros *Burkholderia, Ralstonia, Acidovorax* y *Comamonas* (que se denominaron formalmente *Pseudomonas* pero que pertenecen a las β–proteobacterias).

El subgrupo de las pseudomonas fluorescentes debe su nombre a la producción, por parte de ciertas especies como *P. aeruginosa, P. fluorescens, P. putida, P. syringae, etc.,* de pigmentos que fluorescen cuando son excitados a longitudes de onda bajas.

Las pseudomonas son considerados organismos ubicuos ya que han sido aislados de una gran variedad de hábitats, desde suelos y aguas a alimentos y material clínico, pasando por animales y plantas. Esta ubicuidad puede ser consecuencia de la diversidad de su metabolismo que les permite utilizar un rango muy amplio de compuestos como fuente de carbono.

Dentro de las pseudomonas fluorescentes podemos encontrar tanto especies patógenas de humanos, animales y plantas, por ejemplo *P. aeruginosa, P. syringae* y *P. tolaasii,* como bacterias beneficiosas que viven asociadas a las raíces de las plantas (*P. chlororaphis* y *P. fluorescens*).

La estirpe objeto de este estudio es *Pseudomonas fluorescens* F113 que fue aislada de la rizosfera de remolacha en Irlanda (Shanahan *et al.*, 1992). La capacidad de *P. fluorescens* F113 para producir compuestos con actividad antibiótica y antifúngica, la convierte en una estirpe interesante para biocontrol (Shanahan *et al.*, 1992). También se ha considerado su uso en biorremediación y se han construido cepas que contienen los genes *bph* que le permiten crecer en bifenilo y cometabolizar bifenilos policlorados (PCBs) en asociación con la planta (Brazil *et al.*, 1995; Karlson *et al.*, 1998; Villacieros *et al.*, 2005).

2. LA RIZOSFERA

La rizosfera, que es la porción de suelo en más íntimo contacto con la raíz de las plantas, se caracteriza por presentar una actividad microbiana intensa debido a la gran cantidad de compuestos como aminoácidos, ácidos orgánicos, azúcares,

hormonas, vitaminas, purinas, pirimidinas, etc., exudados por las raíces de las plantas que sirven de nutrientes para estos microorganismos (los exudados radiculares pueden contener hasta un 30% del CO_2 fijado por la planta). De hecho, el número de bacterias que hay en la rizosfera es entre 10 y 1000 veces superior al de bacterias que se encuentran en el suelo no rizosférico (Bazin *et al.*, 1990; Schloter *et al.*, 1997).

De entre estos microorganismos capaces de colonizar la rizosfera los más abundantes son las bacterias y hongos. Las bacterias son especialmente útiles por su susceptibilidad a ser modificadas genéticamente. Las capacidades naturales de estos microorganismos pueden ser alteradas, mejoradas o controladas gracias a técnicas de biología molecular. Algunos de estos organismos modificados genéticamente (OMGs) se usan actualmente como alternativa o de un modo suplementario para reducir el uso de compuestos químicos en agricultura (de Weger *et al.*, 1995; Gerhardson, 2002; Welbaum *et al.*, 2004) y se prevé que su utilización aumente en un futuro próximo.

Las PGPBs (Plant Growth-Promoting Bacteria) se encuentran asociadas a muchas plantas y están normalmente presentes en muchos ambientes. El grupo más estudiado de PGPBs son las PGPRs (Plant Growth-Promoting Rhizobacteria), bacterias que colonizan la superficie de las raíces y la rizosfera (Kloepper & Schroth, 1978) y que son capaces de mejorar el estado de las plantas o de incrementar la productividad de los cultivos (Kloepper *et al.*, 1989). Estas PGPRs pueden clasificarse en tres tipos según sus efectos beneficiosos. Los biofertilizantes mejoran la productividad de los cultivos aumentando la disponibilidad de nutrientes como el nitrógeno y el fósforo para que puedan ser utilizados eficientemente por parte de la planta. Los fitoestimuladores promueven directamente el crecimiento de la planta mediante la producción de hormonas vegetales. Y por último, los agentes biocontroladores que son capaces de proteger las plantas de infecciones por

organismos fitopatógenos, bien sea como antagonistas de estos patógenos, mediante inducción de una respuesta sistémica o competición.

En estos momentos la biofertilización supone aproximadamente el 65% del aporte de nitrógeno a los cultivos en todo el mundo. Dentro de los ejemplos mejor conocidos de biofertilización se encuentran la familia *Rhizobiaceae* y los hongos micorrizógenos.

Entre los más eficientes fijadores de nitrógeno se encuentran cepas bacterianas que pertenecen a los géneros *Rhizobium, Sinorhizobium, Mesorhizobium, Bradyrhizobium, Azorhizobium* y *Allorhizobium*, todas ellas formando una simbiosis específica de huésped con plantas leguminosas en las que se desarrolla un nuevo órgano de la planta, el nódulo. En los últimos años el número de especies noduladoras ha ido aumentando, encontrándose cepas de *Blastobacter*, *Ochrobactrum, Devosia* y *Phyllobacterium* e incluso de β-proteobacterias como *Burkholderia* y *Ralstonia* (Barrett & Parker, 2005; Chen *et al.*, 2001; Chen *et al.*, 2003; Moulin *et al.*, 2001; Rasolomampianina *et al.*, 2005). También se han descubierto recientemente fijadores de nitrógeno endofíticos como es el caso de *Azoarcus* que es capaz de fijar N_2 en herbáceas y arroz (Reinhold-Hurek & Hurek, 1998; Steenhoudt & Vanderleyden, 2000) y *Herbaspirillum* y *Acetobacter* que lo hacen en caña de azúcar (Okkon *et al.*, 1998).

Los hongos micorrizógenos actúan ampliando la rizosfera, incrementando enormemente el alcance del sistema radicular de la planta (Bonfante & Perotto, 1995). Esta simbiosis de la raíz con el hongo se denomina micorriza y afecta a la gran mayoría de las especies de plantas terrestres (Bonfante & Perotto, 1995; Kistner & Parniske, 2002). En este caso el hongo obtiene fuentes de carbono del huésped mientras ayuda a la planta en la captación de nutrientes minerales, principalmente fósforo y agua, del suelo. La micorrización y en consecuencia la captación de nutrientes por parte de la planta también se puede aumentar por la

acción de ciertas bacterias entre las que se encuentran las pseudomonas (Frey-Klett *et al.*, 1997; Garbaye, 1994).

El efecto de la fitoestimulación se debe a la secreción de fitohormonas como auxinas, citoquinas y giberelinas por parte ciertas rizobacterias como es el caso de *Azospirillum* y *Pseudomonas* (Patten & Glick, 2002; Steenhoudt & Vanderleyden, 2000). De todas estas hormonas, el ácido indolacético (AIA) es cuantitativamente el más importante. El AIA provoca un aumento del sistema radicular del huésped permitiendo un aumento en la captación mineral (Patten & Glick, 2002; Steenhoudt & Vanderleyden, 2000).

Hay muchas enfermedades que afectan a cultivos de gran importancia económica como el trigo, la remolacha, el algodón o el tabaco y que están causadas por bacterias y hongos fitopatógenos. Como alternativa al uso de compuestos químicos, que muchas veces no son capaces de combatir ciertas enfermedades eficientemente, se están utilizando microorganismos biocontroladores entre los que se encuentran bacterias del género *Bacillus*, *Pseudomonas* y *Streptomyces* y hongos del género *Trichoderma*, *Gliocladium* y *Fusarium* (Alabouvette & Couteaudier, 1992; Bolwerk *et al.*, 2005; Chin-A-Woeng *et al.*, 1998; Dekkers *et al.*, 2000; Lugtenberg & Bloemberg, 2004; Schippers *et al.*, 1987). Las bacterias pueden controlar estos microorganismos patógenos mediante la producción de metabolitos con actividad antibiótica y antifúngica (Bolwerk *et al.*, 2003; Chin-A-Woeng *et al.*, 2003; Gutterson, 1990; Pfender *et al.*, 1993; Shanahan *et al.*, 1992; Thomashow & Weller, 1988), la inducción de resistencia sistémica (ISR) a través de componentes como flagelos, lipopolisacáridos (LPS), sideróforos y 2,4-diacetilfloroglucinol (Hartmann *et al.*, 2004; Iavicoli *et al.*, 2003; Leeman *et al.*, 1996; Maurhofer *et al.*, 1994a; Pieterse *et al.*, 1996; Pieterse *et al.*, 1998; Pieterse *et al.*, 2000; Ton *et al.*, 2002; van Loon *et al.*, 1998; Van Wees *et al.*, 1997), y la competición por nutrientes y nichos (Bolwerk *et al.*, 2003; Ellis *et al.*, 1999; Kamilova *et al.*, 2005; Raaijmakers *et al.*, 1995). Los hongos biocontroladores pueden usar como

mecanismos de acción la ISR (Duijff *et al.*, 1998; Fuchs *et al.*, 1997), la predación y el parasitismo (Harman *et al.*, 2004) y la competición por nichos y nutrientes (Alabouvette, 1986; Bolwerk *et al.*, 2005; Lemanceau & Alabouvette, 1990).

Nuestra estirpe de trabajo, *Pseudomonas fluorescens* F113, produce 2,4-diacetilforoglucinol (DAPG o Phl). Este compuesto tiene especial interés ya que es un fungicida de amplio espectro de actuación, se ha mostrado efectivo frente a la enfermedad del "black root-rot" del tabaco producida por el hongo *Thilabiopsis basicota* (Keel *et al.*, 1992) y del "damping off" causada por el hongo *Pythium ultimum* en la remolacha (Fedi *et al.*, 1997; Shanahan *et al.*, 1992), reduciendo la persistencia de este patógeno en la rizosfera (Fenton *et al.*, 1992). Por esta razón, F113 se considera una estirpe valiosa para el control de esta plaga y ya ha sido liberada voluntariamente en campo (Moenne-Loccoz *et al.*, 2001).

Con respecto a la colonización de la rizosfera, *P. fluorescens* F113 es una colonizadora competente de la remolacha (*Beta vulgaris*) de la que fue aislada (Shanahan *et al.*, 1992), y también se ha comprobado que es una estirpe que coloniza eficientemente las raíces de otras plantas de interés como el guisante (*Pisum sativum*) (Naseby & Lynch, 1999), el tomate (*Lycopersicon esculentum* Mill c.v. Carmello) (Dekkers *et al.*, 2000; Simons *et al.*, 1996), el sauce (*Salix viminalis*) y la alfalfa (*Medicago sativa*) (Villacieros, 2000; Villacieros *et al.*, 2003).

2.1. Factores implicados en la colonización

El estudio del proceso de colonización de la rizosfera es muy importante para la utilización de microorganismos como inoculantes en cualquier aplicación biotecnológica (biocontrol, rizorremediación, etc) ya que la colonización es el modo natural de distribución del agente. De hecho, se ha demostrado que la colonización es un paso limitante y a veces esencial para el biocontrol (Bull *et al.*, 1991; Chin-A-Woeng *et al.*, 2000). De entre todos los microorganismos presentes en la rizosfera

las pseudomonas están siendo utilizadas como microorganismos modelo en estos estudios de colonización debido a que se encuentran entre las bacterias más competentes de la rizosfera (Lugtenberg *et al.*, 2001).

Los primeros estudios para la búsqueda sistemática de mutantes afectados en colonización competitiva de la raíz se hicieron en *P. fluorescens* WCS365. Para ello se realizó una mutagénesis al azar con el transposón Tn5 y se generó un banco de mutantes que fueron utilizados en ensayos de competitividad frente a la estirpe silvestre (Lugtenberg *et al.*, 2001). Este y otros trabajos dieron lugar a identificación de muchos genes y caracteres importantes para la colonización (Tabla 1.1).

Otro hecho importante que dio lugar al mejor entendimiento de la colonización de la rizosfera fue la utilización de métodos de microscopía y de fluorescencia para realizar estudios del patrón de colonización, ya que estos métodos permiten la visualización de células individuales en la rizosfera. Estos estudios revelaron que la distribución de *Pseudomonas* en la rizosfera no es homogénea sino que se encuentran en microcolonias o formando biofilms (Bloemberg *et al.*, 2000; Chin-A-Woeng *et al.*, 1997; Villacieros, 2000), normalmente en las uniones entre las células de la epidermis radicular, lesiones de la superficie epidérmica y lugares donde están emergiendo raíces secundarias (Rovira, 1956; Yao & Allen, 2006). Además la mayor densidad de bacterias está en la base de la raíz y va decreciendo en dirección al ápice radicular donde puede llegar a ser dos órdenes de magnitud menor (Chin-A-Woeng *et al.*, 1997; Loper *et al.*, 1984; Simons *et al.*, 1996).

La típica estrategia de gen-fenotipo requiere la identificación de factores en base a la inactivación de un gen. Esta herramienta, aunque ha sido fundamental en los estudios de colonización, no siempre es apropiada para el análisis de fenotipos más complejos, este es el caso de las bacterias que colonizan la rizosfera, cuyo comportamiento está condicionado por muy diversos rasgos y factores ambientales.

Los recientes avances en las tecnologías de fusión de genes ofrecen una vía alternativa al estudio de fenotipos complejos permitiendo la identificación de genes

Tabla 1.1. Genes de *Pseudomonas* esenciales para la colonización competitiva del ápice radicular

Clasificación	**Gen(es)**	**Función**	**Referencia**
Estructura superficie celular	No identificado	Síntesis del antígeno O del LPS	(Dekkers *et al.*, 1998c)
	colS	Sensor de sistema de dos componentes	(Dekkers *et al.*, 1998a)
	Homólogo a *htrB*	Transferencia ácido graso al lípido A del LPS	(Dekkers *et al.*, 1998c)
	orf222	*Operón orf222-wapQ;* menor permeabilidad de la membrana	(Lugtenberg & Bloemberg, 2004)
	fleQ, fliC, fliS	Flagelos	(Capdevila *et al.*, 2004; de Weger *et al.*, 1987)
	pilA, pilT	Pili tipo IV	(Lugtenberg & Bloemberg, 2004)
	dsbA	Formación de puentes disulfuro	(Mavrodi *et al.*, 2006a)
Defensa bacteriana	Zona reguladora del operón *pot*	Regulación negativa de la captación de putrescina	(Kuiper *et al.*, 2001)
Generación de energía	Homólogo a *mqo*	Malato deshidrogenada del ciclo del ácido cítrico	(Lugtenberg *et al.*, 2001)
	Operón n*uo*	NADH deshidrogenada I; generación de fuerza protón motriz	(Carvajal *et al.*, 2002)
Auxotróficos	*tyrB*	Defecto en competitividad complementado por tirosina, fenilalanina, ácido aspártico y leucina	(Lugtenberg *et al.*, 2001)
	panB	Ketopantoato hidroximetiltransferasa; implicada en biosíntesis de pantotenato (vit B_5)	(Rainey, 1999)
Hierro	*exbB*/*exbD*/*tonB*	Sistema TonB; captación de hierro	(Molina *et al.*, 2005)
Reordenamiento ADN	xerC/sss, *xerD*	Recombinasas específicas de sitio de la familia de las lambda integrasas	(Dekkers *et al.*, 1998b; Martínez-Granero *et al.*, 2005)

con alguna importancia ecológica en base a su contribución positiva a fenotipos específicos. La estrategia de captura de promotores denominada IVET (*in vivo* expression technology) (Mahan *et al.*, 1993; Osbourn *et al.*, 1987) ha supuesto una revolución en la identificación y entendimiento de genes funcionales incluso en ambientes complejos. Este método permite un análisis a gran escala de los promotores que se inducen en las condiciones de estudio a través de la expresión de un gen que es esencial para la supervivencia de la bacteria.

Dentro de las *Pseudomonas* se han realizado diversos estudios de IVET para ver genes que se inducen en la colonización de la rizosfera (Rainey, 1999; Ramos-González *et al.*, 2005), de hojas (Marco *et al.*, 2003; Marco *et al.*, 2005) y de hongos fitopatógenos (Lee & Cooksey, 2000), durante la infección de *Arabidopsis thaliana* (Boch *et al.*, 2002) e incluso en suelo (Silby & Levy, 2004) (Tabla 1.2).

El papel de los flagelos en colonización dio lugar a mucha controversia durante los años ochenta. Howie y col. (1987) y Scher y col. (1988) observaron que mutantes no móviles de *Pseudomonas* no estaban afectados en colonización de la raíz de trigo y soja, respectivamente. Por otro lado, de Weger y col. (1987) encontraron diferentes mutantes inmóviles de *P. fluorescens* WCS374 afectados en la colonización de la raíz de patata. Estudios posteriores, utilizando diferentes *Pseudomonas*, sistemas de suelo y plantas, demostraron que los mutantes inmóviles pertenecen a la clase de mutantes que están más afectados en colonización competitiva (Capdevila *et al.*, 2004; Chin-A-Woeng *et al.*, 2000; Dekkers *et al.*, 1998c; Kamilova *et al.*, 2005; Simons *et al.*, 1996). Incluso se ha visto que la expresión de *fliO*, implicado en la síntesis del aparato de exportación flagelar, aumenta en la rizosfera (Ramos-González *et al.*, 2005). Pero el papel de los flagelos en la colonización de la rizosfera no se debe a una movilidad al azar sino que se

Tabla 1.2. Genes de *Pseudomonas* inducidos durante colonización

Clasificación	Gen(es)	Función	Referencia
Estructura superficie celular	*fliO*	Aparato de exportación flagelar	(Ramos-González *et al.*, 2005)
	algD	Biosíntesis del exopolisacárido alginato	(Ramos-González *et al.*, 2005)
	algA	Biosíntesis del exopolisacárido alginato	(Boch *et al.*, 2002)
	secB	Chaperona de ruta de secreción	(Ramos-González *et al.*, 2005)
	yidC	Inserción de proteínas en la membrana	(Ramos-González *et al.*, 2005)
Reguladores	*colS*/*colR*	Sistema de dos componentes	(Ramos-González *et al.*, 2005)
	copR/*copS*	Sistema de dos componentes implicados en resistencia al cobre	(Rainey, 1999)
	wspE	Regulador quimiotaxis; síntesis de celulosa	(Marco *et al.*, 2005)
Adquisición de nutrientes y metabolismo	*hutT*	Permeasa inducible por histidina	(Rainey, 1999)
	braE-braD	Parte de permeasa para transporte de aminoácidos ramificados	(Rainey, 1999)
	xylA	Xilosa isomerasa	(Rainey, 1999)
	morB, ncr	Reductasa implicada en utilización de compuestos nitrogenados complejos	(Rainey, 1999)
	gnd	6-fosfogluconato deshidrogenada (ruta pentosas fosfato)	(Ramos-González *et al.*, 2005)
	aceE	Componente E1 de la piruvato deshidrogenada (metabolismo central)	(Ramos-González *et al.*, 2005)
	ssuE	Reductasa FMN dependiente de NAD(P)H; metabolismo del azufre	(Marco *et al.*, 2005)
	pUIVS2	Transportador tipo ABC; posible captación de compuestos	(Lee & Cooksey, 2000)
	oprD2	Probable porina de membrana externa; posible captación de compuestos	(Lee & Cooksey, 2000)
Defensa bacteriana y virulencia	*ragC*	Miembro de la familia AcrB/AcrD/AcrF de bombas de extrusión	(Rainey, 1999)

Tabla 1.2. Continuación

	rosA	Proteína transmembrana; resistencia a fosmidomicina	(Rainey, 1999)
	syrE	Siringomicina sintetasa	(Boch *et al.*, 2002; Marco *et al.*, 2005)
	sylE	Posible transportador de siringolina A	(Marco *et al.*, 2005)
	varios genes	Sistema de secreción tipo III	(Boch *et al.*, 2002)
Reordenamiento ADN	*sss*/*xerD*	Recombinasas específicas de sitio de la familia de las lambda integrasas	(Marco *et al.*, 2003; Marco *et al.*, 2005; Martínez-Granero *et al.*, 2005)
	orfC	Elemento de inserción ISI50	(Marco *et al.*, 2005)

debe a una movilidad quimiotáctica, ya que mutantes en el gen *cheA* de varias estirpes de *P. fluorescens* (WCS365, OE 28.3, SBW25 y F113) presentaban el mismo fenotipo de deficiencia en la colonización que aquellos mutantes que carecían de flagelos (de Weert *et al.*, 2002). Esta importancia de la quimiotaxis no sólo se ha observado en *Pseudomonas*. En el fitopatógeno *Ralstonia solanacearum* se ha visto que mutantes no quimiotácticos (*cheA*$^-$ y *cheW*$^-$) que aún presentan movilidad no son capaces de competir con la cepa silvestre (Yao & Allen, 2006). Así pues, parece que la quimiotaxis tiene un papel muy importante en la interacción entre plantas y bacterias (Brencic & Winans, 2005; Vande Broek & Vanderleyden, 1995).

En *P. aeruginosa*, los pili tipo IV que median en el contacto inicial entre la bacteria y la superficie de células epiteliales (Hahn, 1997), están implicados en un tipo de movimiento único denominado *twitching* (Darzins & Russell, 1997) y también intervienen en adhesión a superficies abióticas y formación de biopelículas o *biofilms* (O'Toole & Kolter, 1998). Aunque se ha visto que varios fitopatógenos presentan pili tipo IV, hay poco documentado sobre su papel en adhesión a las superficies de la planta. En *Xantomonas campestris* pv. *hyacinthi* y *P. syringae* pv. *tomato* DC3000 se ha visto que facilitan la adhesión a hojas (Ojanen-Reuhs *et al.*, 1997; Roine *et al.*, 1998). Los pili tipo IV también juegan un papel en colonización

de plantas y hongos por parte de la bacteria endofítica *Azoarcus* (Dörr *et al.*, 1998; Steenhoudt & Vanderleyden, 2000). Mutantes en *pilA* (no presenta pili) y *pilT* (pili incapaces de retraerse) de *P. fluorescens* WCS365 están afectados, aunque no de una forma drástica, en la colonización competitiva del ápice radicular (Lugtenberg & Bloemberg, 2004); probablemente el papel que juegan los pili tipo IV en la colonización competitiva del ápice de la raíz del tomate sea a través del movimiento tipo *twitching*.

Los mutantes que presentan anomalías en el patrón de LPS, en particular en el antígeno O, son muy frecuentes entre los mutantes afectados en colonización competitiva de la rizosfera (Dekkers *et al.*, 1998c). La interpretación de estos defectos en colonización de estos mutantes que no presentan antígeno O es complicada porque muestran serios problemas de crecimiento tanto en medios de cultivo como con exudados radiculares (Dekkers *et al.*, 1998c; Lugtenberg *et al.*, 2001). Sin embargo, entre estos mutantes se encuentra el PCL1205 que tiene un antígeno O más pequeño y una tasa de crecimiento normal, pero al igual que los demás está afectado en colonización (Dekkers *et al.*, 1998c). Esto sugiere que además del posible papel en la tasa de crecimiento, el antígeno O por sí mismo, de alguna otra forma, está implicado en la colonización de la rizosfera.

Aquellos factores que afectan a la integridad de la membrana parece que también son importantes para la colonización. Se ha descrito que una mutación en el sistema de dos componentes *colR/colS* de *P. fluorescens* afecta a la colonización de la rizosfera de diversas plantas (Dekkers *et al.*, 1998a), corroborando el hecho de que este sistema se induce en *P. putida* KT2440 durante la colonización de la rizosfera de maíz (Ramos-González *et al.*, 2005). Este mutante es más sensible a polimixina B, la cuál elimina el LPS de la membrana externa, y está afectado en crecimiento competitivo en distintas fuentes de carbono. Estas observaciones apuntan a un posible defecto en la permeabilidad de la membrana externa. De hecho, un mutante en el operón *orf222-wapQ* adyacente a *colR/colS* también está

afectado en colonización competitiva. Parece que *colR/colS* está regulando la expresión de *wapQ* (que codifica una heptosa quinasa) y esta actividad quinasa es necesaria para una apertura completa de los poros de la membrana externa, permitiendo a la célula competir óptimamente por los nutrientes en la rizosfera (Lugtenberg & Bloemberg, 2004). La integridad de la envuelta celular puede tener también un papel crucial en la colonización de la rizosfera porque se ha observado que durante la colonización de la raíz de maíz por *P. putida* KT2440 hay una inducción del operón *algD-8-44-KEGXLIJFA*, responsable de la biosíntesis del exopolisacárido (EPS) alginato (Ramos-González *et al.*, 2005). Y además, en *P. syringae* pv. *syringae* mutantes incapaces de producir alginato tienen afectados la habilidad de colonizar los tejidos de la planta (Yu *et al.*, 1999). La inducción en la rizosfera de *secB*, que codifica una chaperona implicada en rutas de secreción, y de *yidC*, que codifica una proteína cuya función es insertar proteínas de membrana, refuerza la importancia que tiene la envuelta celular bacteriana en la colonización (Ramos-González *et al.*, 2005).

Recientemente se ha visto que un mutante en el gen *dsbA* de *P. fluorescens* Q8r1-96 está afectado en la colonización de la rizosfera de trigo (Mavrodi *et al.*, 2006a). Probablemente el efecto sea indirecto ya que *dsbA* en bacterias Gram negativas codifica una enzima periplásmica que cataliza la formación de puentes disulfuro (Bardwell *et al.*, 1991). Su implicación puede ser a través de la biogénesis de proteínas que se secretan y de estructuras de la superficie celular que sean importantes para la colonización. Entre los efectos de mutaciones en *dsbA* en otros microorganismos están las deficiencias en patogenicidad y competitividad asociadas con la pérdida de movilidad y la incapacidad de producir fimbrias y secretar enzimas (Dailey & Berg, 1993; Kloek *et al.*, 2000; Shevchik *et al.*, 1995).

Además de los caracteres externos ya nombrados, existen funciones metabólicas que al ser alteradas pueden limitar una buena colonización. Se han documentado mutantes auxotróficos para distintos compuestos como vitaminas y

aminoácidos. En *P. fluorescens* WCS365 y SBW25 se han descrito mutantes auxotróficos para vitaminas (B_1 y B_5, respectivamente) que no compiten bien en la rizosfera (Rainey, 1999; Simons *et al.*, 1997). No sólo en *Pseudomonas* se ha observado esta relación entre auxotrofía y colonización. En *Sinorhizobium meliloti* también se ha visto un mutante auxotrófico para biotina que no es un buen competidor en la rizosfera de alfalfa (Streit & Phillips, 1997). Al igual que el mutante auxotrófico para tiamina de WCS365, se aislaron cinco mutantes que no podían sintetizar algún aminoácido y que eran incapaces de colonizar eficientemente la raíz del tomate, tanto solos como en competición con la cepa silvestre. La adición del aminoácido apropiado hizo que se restableciese la colonización de los mutantes (Simons *et al.*, 1997). También, durante la colonización de la rizosfera de remolacha por *P. fluorescens* SBW25 se ha visto mediante IVET una inducción en dos genes que parece que forman parte de dos sistemas de captación de aminoácidos (Rainey, 1999).

Puesto que los exudados de la raíz son la principal fuente de nutrientes para los microorganismos de la rizosfera (Van Overbeek & Van Elsas, 1995), la competencia en la rizosfera implica que los microorganismos estén bien adaptados para su utilización (Lugtenberg *et al.*, 1999). Así pues, el mutante PCL1085 de *P. fluorescens* WCS365, que presenta una mutación en la zona promotora de un gen que codifica una malato deshidrogenasa, no crece bien utilizando ácido málico, succínico y cítrico (compuestos mayoritarios exudados por la raíz del tomate) como fuente de energía y no coloniza competitivamente la raíz de esta planta (Lugtenberg & Bloemberg, 2004). De hecho, la búsqueda de colonizadores competitivos eficientes selecciona entre otros factores un eficiente aprovechamiento de los principales compuestos exudados por la raíz (Kamilova *et al.*, 2005; Kuiper *et al.*, 2002). De ahí que sea normal encontrar genes relacionados con la adquisición de nutrientes y con el metabolismo de azúcares y derivados entre aquellos que se inducen en la rizosfera (Rainey, 1999; Ramos-González *et al.*, 2005).

En los exudados, además de una gran cantidad de nutrientes, también pueden encontrarse ciertos compuestos tóxicos como es el caso de las poliaminas. Estos compuestos, a altos niveles, pueden retardar el crecimiento bacteriano mediante una bacteriostasis transitoria, haciendo que estas bacterias sean menos competitivas en la rizosfera (Kuiper *et al.*, 2001). Avalando esta teoría, Espinosa-Urgel y col. (2000) han encontrado entre una serie de mutantes de *P. putida* afectados en colonización de semilla de maíz uno que está mutado en una posible bomba de extrusión que podría estar implicada en la protección contra toxinas presentes en los exudados. De hecho, en otros trabajos se han encontrado genes inducidos en la rizosfera que forman parte de bombas de extrusión y se piensa que intervienen en la protección de la bacteria frente a metabolitos dañinos producidos por la planta o por otros microorganismos circundantes (Rainey, 1999).

El hierro es un elemento esencial para el crecimiento de todos los organismos. La escasez de hierro biodisponible en el suelo y en la rizosfera genera una fuerte competición por este vital elemento (Loper & Henkels, 1997). La producción de sideróforos como la pioverdina, responsable de la captación de hierro en *Pseudomonas*, e incluso la utilización de sideróforos heterólogos se han revelado fundamentales para una buena actuación en la rizosfera (Loper & Henkels, 1997; Loper & Henkels, 1999; Mirleau *et al.*, 2000; Raaijmakers *et al.*, 1995). Datos que se correlacionan con el hecho de que un mutante en el sistema TonB en *P. putida* KT2440, incapacitado para la internalización del complejo sideróforo-Fe, presenta también una clara desventaja en la colonización de la semilla y la rizosfera del maíz (Molina *et al.*, 2005).

Existen otros elementos traza en la rizosfera que son esenciales en cantidades pequeñas pero que son tóxicos en exceso. El hecho de haber encontrado entre genes que se inducen en la rizosfera de *P. fluorescens* SBW25 el sistema de dos componentes *copS*/*copR*, que confiere resistencia al cobre, da a entender que

estos elementos traza pueden llegar a ser perjudiciales y resalta la importancia del mantenimiento de una homeostasis correcta (Rainey, 1999).

También se ha encontrado que una mutación en un gen homólogo a *nuoD*, que codifica una NADH deshidrogenada I, afecta negativamente a la colonización competitiva de la rizosfera del tomate (Carvajal *et al.*, 2002; Dekkers *et al.*, 1998c). La NADH deshidrogenasa I está implicada en la generación de la fuerza protón motriz, la cuál puede utilizarse para la captación de nutrientes, la generación de ATP y la rotación del flagelo. Parece claro que la expresión de *nuo* en la rizosfera es importante ya que entre los factores clave para que se de una buena competencia en la rizosfera se encuentran una eficiente captación de nutrientes y la movilidad quimiotáctica.

Uno de los mutantes afectados en colonización competitiva de *P. fluorescens* WCS365 más interesantes es el mutante PCL1233. En esta cepa el gen que afecta a la colonización es un homólogo a *xerC/sss* (Dekkers *et al.*, 1998b), miembro de las recombinasas específicas de sitio de la familia lambda integrasa. En otros trabajos más recientes se corrobora este hallazgo (Martínez-Granero *et al.*, 2005; Mavrodi *et al.*, 2006b) y se añade otra recombinasa específica de sitio denominada *xerD* a la lista de genes implicados en el proceso de colonización competitiva (Martínez-Granero *et al.*, 2005), de hecho se ha visto que estas dos recombinasas se inducen durante la colonización de diversas partes de la planta (Marco *et al.*, 2003; Marco *et al.*, 2005; Martínez-Granero *et al.*, 2005). Los miembros de estas recombinasas específicas de sitio promueven la recombinación entre dos fragmentos homólogos de ADN (Sadowski, 1986). Tales recombinaciones pueden jugar un papel en variación de fase. Por ejemplo, las recombinasas específicas de sitio pueden regular la expresión de las fimbrias en *Escherichia coli* (Abraham *et al.*, 1985), la producción de dos tipos de flagelinas en *Salmonella typhymurium* (Zieg *et al.*, 1977) y la variación antigénica de superficie en *Mycoplasma penetrans* (Horino *et al.*, 2003). Esto sugiere que la incapacidad de

producir reordenamientos de ADN puede afectar a uno o más caracteres que se han descrito importantes para la colonización de la raíz. Además estos reordenamientos parecen ser importantes en la formación de subpoblaciones que le permiten a la bacteria adaptarse a cambios repentinos del ambiente (Dybvig, 1993).

3. VARIACIÓN DE FASE (FENOTÍPICA)

La variación de fase fue definida por Saunders y col. (2003) como un proceso que produce cambios fenotípicos reversibles de alta frecuencia que están mediados por mutaciones, reorganizaciones o modificaciones del ADN. Este fenómeno ha sido descrito en muchas especies bacterianas, principalmente dentro de las Gram negativas (revisado en Henderson y col. (1999) y van den Broek y col. (2005a)).

Muchas especies bacterianas utilizan la variación de fase para generar una diversidad en la población muy importante para la adaptación al nicho en el que se encuentran (Dybvig, 1993). La mayoría de los ejemplos de variación de fase o variación fenotípica se han descrito en el contexto de las interacciones huésped-patógeno como mecanismos que permiten al patógeno escapar del sistema inmune del huésped (Borst, 2003). Sin embargo, estudios recientes describen la variación de fase en un contexto mucho más amplio, implicándola en la producción de exo-enzimas y metabolitos secundarios, la colonización de la raíz y el control biológico de agentes fitopatógenos (Achouak *et al.*, 2004; Chabeaud *et al.*, 2001; Chancey *et al.*, 2002; Sánchez-Contreras *et al.*, 2002; van den Broek *et al.*, 2003).

La variación de fase afecta principalmente a componentes superficiales de la bacteria como antígenos de membrana, flagelos, fimbrias, etc., que intervienen en movilidad y adhesión a superficies, y causan variaciones morfológicas en las colonias que son de fácil detección (Henderson *et al.*, 1999). Los ejemplos más estudiados son la variación de fimbrias tipo 1 en *Escherichia coli* (Abraham *et al.*,

1985), la variación flagelar en *Salmonella typhimurium* (Zieg *et al.*, 1977) y la variación de antígenos de superficie en diferentes patógenos del género *Neisseria* (Meyer & Hill, 2003).

3.1. Mecanismos de variación de fase

Desde el punto de vista molecular, el fenómeno de variación de fase se debe a diversos mecanismos que se pueden agrupar en programados y no programados (Borst, 2003). La variación programada implica conversiones reguladas de ADN como resultado de errores de fase durante la replicación del ADN que producen cambios en el marco de lectura durante la transcripción (Sarkari *et al.*, 1994) o la traducción (Park *et al.*, 2000), reordenamientos genómicos (Abraham *et al.*, 1985) o cambios epigenéticos debidos a metilaciones diferenciales (van der Woude *et al.*, 1996). La variación de fase no programada provoca alteraciones del ADN debido a la acumulación de errores durante la replicación, reparaciones defectuosas en el ADN o recombinación entre genes que no son idénticos (Borst, 2003).

3.1.1. Cambio de fase

Los cambios de fase se dan en secuencias cortas repetidas que regulan la expresión génica tanto a nivel transcripcional como de traducción. Este ADN repetitivo puede estar formado por una región homopolimérica o varias repeticiones multiméricas que pueden ser homogéneas, heterogéneas o degeneradas (Henderson *et al.*, 1999). La estabilidad de estas regiones repetidas está influenciada por varios factores: (i) El número de repeticiones, un aumento en el número de repeticiones supone un aumento en la tasa de mutación; (ii) La longitud de la unidad repetida, cuando la unidad de repetición es menor de 5 pares de bases la tasa de mutación se suprime por el sistema de reparación de errores de apareamiento dirigido por metilación (MMR); (iii) La composición nucleotídica de la secuencia repetida; (iv) La replicación del ADN y procesos relacionados con la replicación como la

corrección de errores ("proofreading"); (v) La transcripción del ADN; (vi) El sistema MMR, que aumenta la estabilidad de las regiones repetidas (Bayliss *et al.*, 2002; Bayliss *et al.*, 2004a; Bayliss *et al.*, 2004b; Levinson & Gutman, 1987; Lovett & Feschenko, 1996).

Las repeticiones se encuentran en la región promotora o dentro de la región codificante del gen y alteran la expresión de dicho gen cambiando el número de repeticiones (Fig. 1.1). Este número de repeticiones varía a través de un mecanismo independiente de RecA en el que se forma una región de triple cadena en la zona de repetición dando como resultado otra región de cadena simple que induce el cambio de fase (Belland, 1991; Henderson *et al.*, 1999).

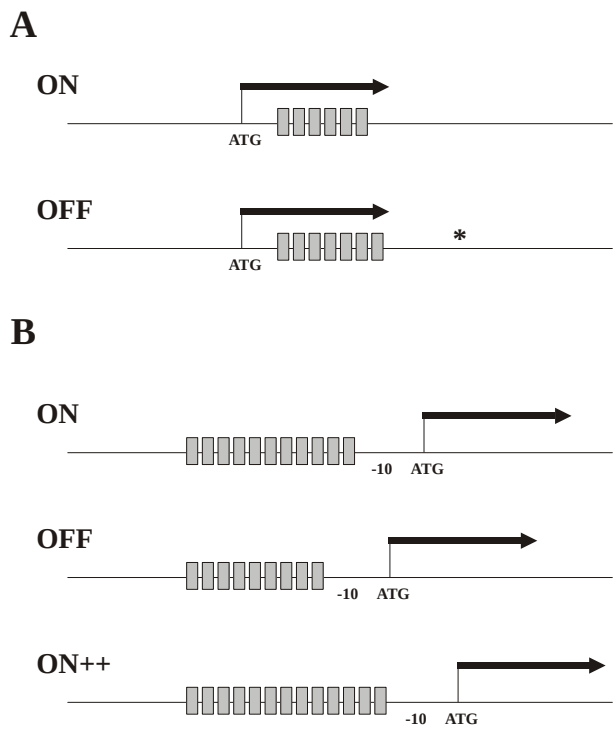

Figura 1.1. Modelo de variación de fase por cambio de fase. (A) A nivel de traducción. El asterisco indica un codón de parada prematuro. (B) A nivel de transcripción. ON++ indica un aumento en nivel de expresión con respecto a ON.

La alteración del número de repeticiones dentro de la región codificante puede dar lugar a una parada prematura de la traducción con motivo del cambio de fase de lectura (Fig. 1.1A). Por ejemplo, la regulación de la expresión de los genes *opa* (adhesinas que intervienen en la interacción bacteria-huésped) de *Neisseria gonorrhoeae* y *Neisseria meningitidis*. El cambio ON◄►OFF se debe a cambios en el número de repeticiones pentaméricas (5´-CTCTT-3´) que se encuentran en la región del péptido señal (Stern *et al.*, 1986). Este mismo sistema también se ha descrito para el gen *bvgS*, que codifica un traductor de señal de un sistema de dos componentes implicado en virulencia, de *Bordetella pertussis* (Stibitz *et al.*, 1989). Parece que este sistema de cambio de fase en la zona codificante está mucho más extendido y es más importante que el cambio de fase a nivel transcripcional, de hecho el análisis del genoma de *Helicobacter pylori* muestra la presencia de 27 genes implicados en biosíntesis de LPS, proteínas de superficie celular y sistemas de modificación del ADN que presentan repeticiones en sus zonas codificantes (Saunders *et al.*, 1998).

Como ya se mencionó con anterioridad el mecanismo de cambio de fase puede regular la expresión de un gen a nivel transcripcional. Esta regulación está mediada por la presencia de repeticiones en la zona promotora del gen, la variación del número de repeticiones da lugar a un aumento o a una disminución de la expresión debido al cambio en la longitud de la zona espaciadora que se encuentra entre la región -10 y -35 (Fig. 1.1B). La proteína de membrana externa Opc de *N. meningitidis* presenta este tipo de regulación. Sin embargo, el gen *opc* no sólo presenta la variación de fase ON◄►OFF sino que también exhibe cambios en los niveles de expresión (Sarkari *et al.*, 1994).

3.1.2. Reordenamientos genómicos

Los mecanismos de variación de fase debidos a reorganizaciones genómicas ocurren en sitios específicos en el genoma, siendo estos reordenamientos al menos

parcialmente programados. La recombinación específica de sitio y la recombinación homóloga general son algo diferentes. Los eventos de recombinación homóloga general necesitan secuencias que presenten una homología bastante alta, en este caso la recombinación tiene lugar en cualquier parte dentro de la región de homología. La frecuencia de recombinación aumenta cuanto más grande sean las regiones homólogas. Para esta recombinación homóloga son necesarias proteínas de la ruta general de recombinación como por ejemplo RecA. Por el contrario, en los eventos de recombinación específica de sitio las secuencias son cortas y el entrecruzamiento ocurre en un punto específico dentro de dicha región. Además, esta recombinación específica de sitio requiere la acción de una recombinasa específica y uno o varios cofactores que son independientes de la ruta general de recombinación (Dybvig, 1993).

Las inversiones de ADN específicas de sitio dan lugar a cambios ON◄► OFF en genes que se encuentran localizados dentro o al lado de la región invertible. Estos cambios alteran la relación espacial de los promotores o de los elementos reguladores respecto a los genes que afectan. Entre los ejemplos mejor caracterizados de variación debida a inversiones están la variación de fase de la fimbria tipo 1 en *Escherichia coli* (Abraham *et al.*, 1985; McClain *et al.*, 1991; McClain *et al.*, 1993) y del flagelo en *Salmonella typhimurium* (Zieg *et al.*, 1977).

La variación de fase de la fimbria tipo 1 en *E. coli* se debe a la inversión de un fragmento de ADN de 314 pb que contiene el promotor del gen *fimA* (subunidad estructural de la fimbria) de forma que en la orientación ON el promotor está en la posición correcta para transcribir *fimA* y en la orientación OFF no hay transcripción (Fig. 1.2A). La inversión de este segmento depende de dos recombinasas específicas de sitio denominadas FimB y FimE y de tres proteínas ayudantes (H-NS, IHF y Lrp) (Blomfield *et al.*, 1993; Blomfield *et al.*, 1997). FimB y FimE actúan de forma independiente, FimE preferentemente invierte el promotor de ON a

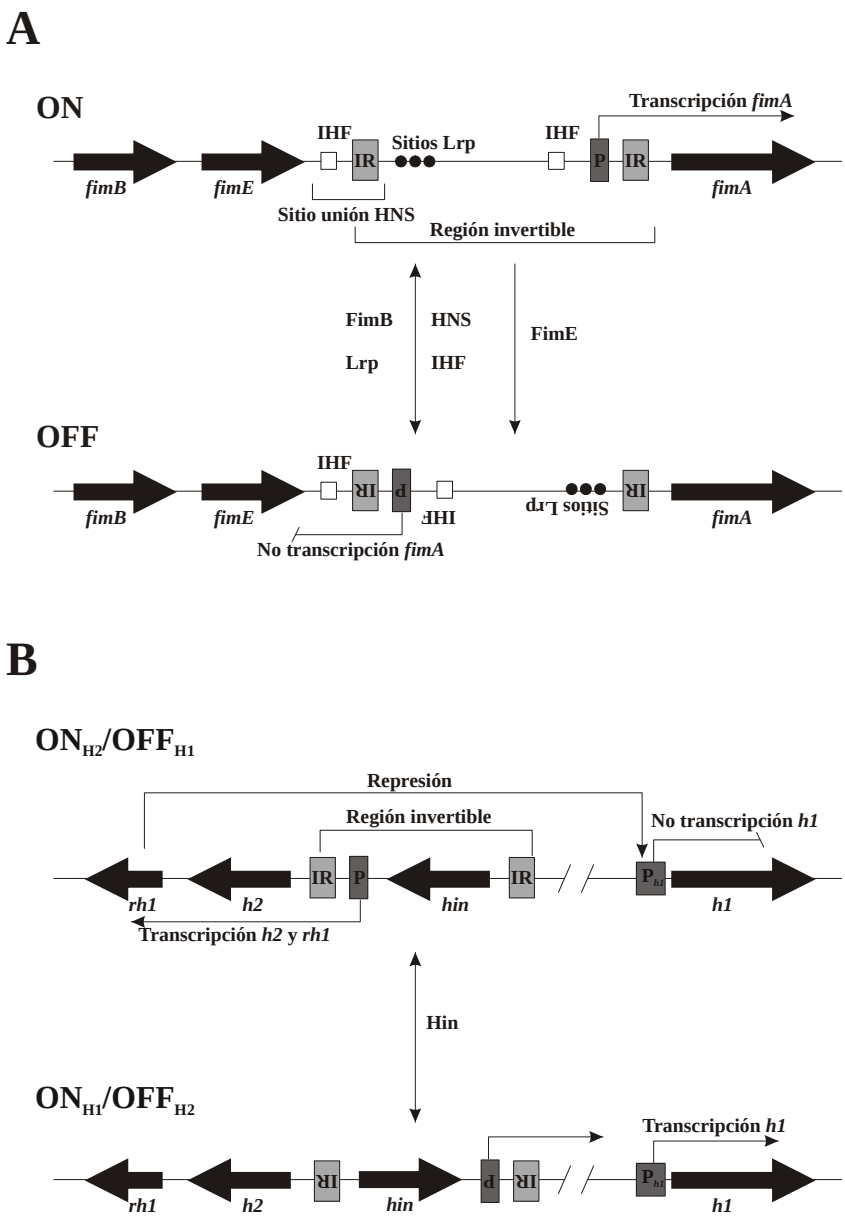

Figura 1.2. Modelo de variación de fase inducida por reordenamiento genómico. (A) Variación de fase de fimbria tipo 1 en *E. coli*. (B) Variación de fase de la flagelina H1 y H2 de *S. typhimurium*. IR indica secuencia repetida invertida, P indica promotor.

OFF (Stentebjerg-Olesen *et al.*, 2000), mientras que FimB puede cambiar el promotor de ON a OFF y viceversa (McClain *et al.*, 1991).

Al contrario que el sistema *fim* de *E. coli*, que presenta un cambio ON↔OFF, los flagelos de *Salmonella* muestran un fenómeno de variación de fase más complejo ($ON_{H2}/OFF_{H1} \leftrightarrow ON_{H1}/OFF_{H2}$) (Fig. 1.2B). La variación flagelar de *S. typhimurium* da lugar a expresión de dos tipos de flagelo que son antigénicamente diferentes, H1 y H2. En la configuración ON_{H2}/OFF_{H1}, la orientación del promotor permite la expresión de la flagelina H2 y del represor Rh1, que a su vez reprime la transcripción del gen *h1* (que codifica para la flagelina H1). Cuando la recombinasa específica de sitio Hin produce la inversión tanto la flagelina *h2* como el represor *rh1* dejan de transcribirse permitiendo, en consecuencia, la expresión de la flagelina *h1* (Zieg *et al.*, 1977).

Además de las recombinaciones específicas de sitio, la recombinación homóloga es también un mecanismo muy importante a la hora de generar diversidad en procariotas. Este mecanismo de variación a través de recombinación homóloga puede dar lugar a inversiones, delecciones, duplicación génica y transferencia de genes usando copias silenciosas (Borst, 2003). Uno de los ejemplos mejor estudiados dentro de esta variación fenotípica dependiente de RecA es la variación del pili tipo IV en *N. gonorrhoeae* (Mehr & Seifert, 1998; Seifert, 1996).

3.1.3. Metilación diferencial

La variación debida a metilación diferencial es diferente de los mecanismos de variación de fase descritos hasta ahora. Este tipo de variación es epigenética ya que el fenotipo cambia pero el genotipo no (van der Woude *et al.*, 1996). En este caso la integridad del genoma se mantiene y lo que cambia es la actuación de varias proteínas reguladoras que alteran la transcripción.

La deoxiadenosina metilasa (Dam) de *E. coli* se une a las secuencias GATC y metila la adenosina en la posición N^6. Normalmente, la metilación supone un mecanismo de regulación para reparación del ADN, protección frente a enzimas de restricción y marcaje de dianas y tiempos en ciertos eventos celulares (Marinus, 1996). Sin embargo, algunos de estos sitios, que se encuentran en regiones implicadas en regulación génica, pueden protegerse diferencialmente de la metilación inhibiendo o facilitando la unión de proteínas reguladoras a sus dianas y por consiguiente alterando la expresión del gen que regulan (van der Woude *et al.*, 1996).

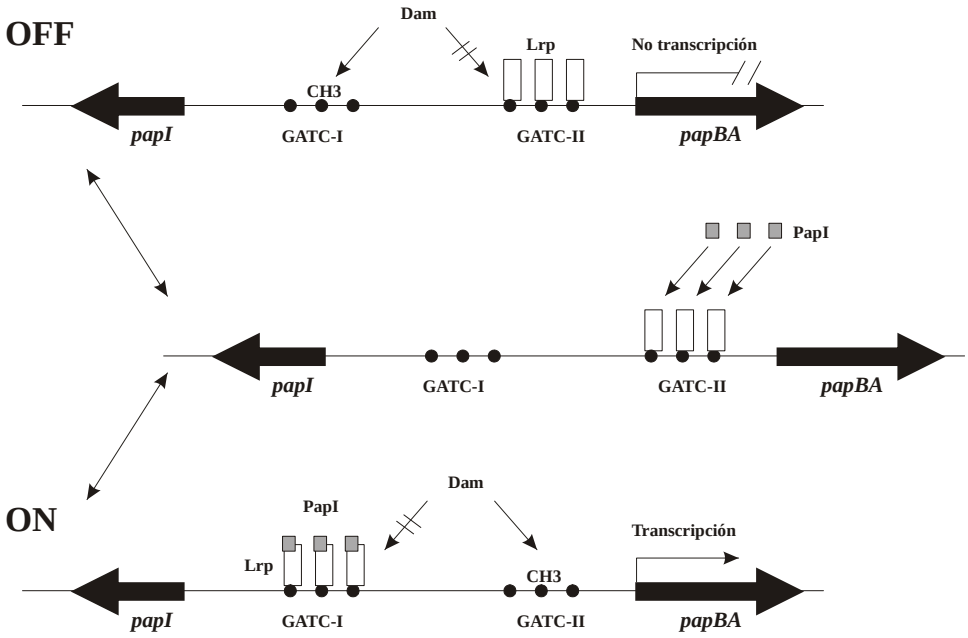

Figura 1.3. Modelo de variación de fase del pili P en *E. coli* a través de metilación diferencial. CH3 indica la metilación del sitio GATC.

La regulación del pili P o Pap (pili asociado a pielonefritis) en cepas uropatogénicas de *E. coli* es uno de lo mecanismos de variación de fase debida a metilación más caracterizado. La regulación viene dada por la metilación

diferencial de dos sitios GATC (GATC-I y GATC-II) que se encuentran en la región reguladora de dos operones divergentes (*papI* y *papBA*). La metilación se regula mediante la competición entre la metilasa Dam y la proteína Lrp que es capaz de proteger los sitios de metilación, la afinidad de Lrp por un sitio u otro depende de la interacción con PapI (Nou *et al.*, 1995). Cuando GATC-I está metilado y GATC-II no, las células están en fase OFF y cuando es al contrario están en ON (van der Woude *et al.*, 1996) (Fig. 1.3).

3.1.4. Variación no programada

La variación no programada depende de la introducción de mutaciones como consecuencia de una replicación imperfecta. Los virus utilizan estas variaciones al azar para eludir el sistema inmune del huésped, permitiéndoles establecer una infección crónica. El lado negativo de un mecanismo que favorece la diversificación en base a replicaciones imperfectas es la alta tasa de mutaciones que conlleva. Los organismos superiores no se pueden permitir descuidar de esta forma la replicación del ADN, por este motivo han desarrollado mecanismos que pueden controlar la tasa de mutación.

Algunos estudios sugieren que los ambientes o condiciones restrictivas pueden favorecer un incremento transitorio en la tasa de mutación a través de la disminución en la fidelidad de la replicación del ADN (inducción de ADN polimerasas propensas a error) o en la actividad de los sistemas de reparación (Borst, 2003; Denamur & Matic, 2006; Kivisaar, 2003; Schofield & Hsieh, 2003; Tegova *et al.*, 2004) (Fig. 1.4). Aunque también se han descrito casos en los que las condiciones ambientales estresantes incrementan la frecuencia de transposición de elementos móviles (Chao *et al.*, 1983; Chao & McBroom, 1985; Ilves *et al.*, 2001; Shapiro, 1997).

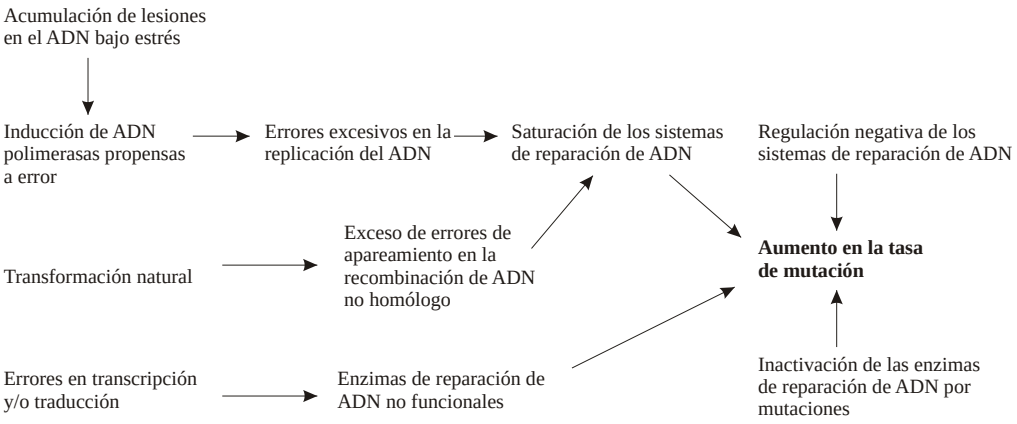

Figura 1.4. Mecanismos que aumentan la tasa de mutación en condiciones de estrés. Adaptación de Kivisaar (2003).

E. coli tiene cinco ADN polimerasas, de las cuáles tres (pol II, pol IV y pol V) se inducen como parte del sistema SOS en respuesta a daños en el ADN (Goodman, 2002). De hecho, la mutación de estas tres polimerasas hace que no crezcan bien en competición con la cepa parental (Yeiser *et al.*, 2002). Estos datos indican que estas polimerasas (II, IV y V) son importantes para la mutagénesis en fase estacionaria ya que la mayor acumulación de mutaciones resultantes de la actividad de estas polimerasas confiere una ventaja en competitividad a un conjunto de células dentro de la población durante fase estacionaria, permitiendo una mejor adaptación a estas condiciones restrictivas.

La eficiencia de la reparación del ADN es un factor muy importante que puede controlar la frecuencia de mutaciones. En muchos casos, el fenotipo hipermutador se induce por la inactivación de genes que codifican enzimas que reparan el ADN (Miller, 1998; Oliver *et al.*, 2002; Saumaa *et al.*, 2002). De hecho, los mutantes afectados en el sistema de reparación MMR (*mutS*, *mutL*, *mutH*) se encuentran entre las cepas que presentan este fenotipo hipermutador ya que pueden

aumentar unas 100 veces la tasa de transición (G:C◄►A:T), 1000 veces la tasa de cambios de fase y entre 10 y 1000 veces la tasa de reordenamiento genómico.

La mayoría de mutaciones suelen ser deletéreas, de ahí que la tasa de mutaciones espontáneas se mantenga a niveles bajos. Sin embargo, bajo ciertas circunstancias, dentro de una población natural de bacterias pueden aparecer ciertos fenotipos con un fuerte carácter mutador (Oliver *et al.*, 2000; Richardson *et al.*, 2002). La adquisición de alelos favorables es más frecuente en los hipermutadores que en los no mutadores y de ahí que se pueda producir un aumento en la frecuencia de aparición de estos fenotipos siempre y cuando los beneficios que suponen los nuevos alelos superen el coste de ser un hipermutador (Taddei *et al.*, 1997). Para evitar el incremento de las mutaciones deletéreas dentro de las tasas globales de mutación que acompañan a un fenotipo mutador, muchas bacterias (por ejemplo, *Haemophilus influenzae*, *Helicobacter pylori*, *Neisseria meningitidis*) han desarrollado mecanismos para incrementar la frecuencias de mutación en aquellos genes que son importantes para la interacción bacteria-huésped, evasión del sistema inmune, etc. (Bayliss *et al.*, 2001; Borst, 2003; Metzgar & Wills, 2000; Moxon *et al.*, 1994). En el contexto de la variación de fase, las mutaciones que se acumulan en estas regiones suelen ser pequeñas delecciones, errores de apareamiento y duplicaciones (Han *et al.*, 1997; Sánchez-Contreras *et al.*, 2002; van den Broek *et al.*, 2005b; Waite *et al.*, 2003).

3.2. Variación de fase (fenotípica) en *Pseudomonas*

Las *Pseudomonas* son conocidas por su habilidad para colonizar múltiples hábitats y por su excelente capacidad para adaptarse rápidamente a nuevos ambientes. En un ambiente espacialmente estructurado, la población bacteriana diverge rápidamente, dando lugar a la aparición de distintos morfotipos adaptados a cada nicho específico (Déziel *et al.*, 2001; Rainey & Travisano, 1998).

En *P. aeruginosa*, la variación de fase regula la expresión del epítopo fosfatidilcolina de una proteína de 43 KDa que parece que puede tener un papel en patogenicidad (Weiser *et al.*, 1998). La aparición de variantes de fase en *P. aeruginosa* también se ha relacionado con movilidad y formación de biopelículas a través de la regulación de la expresión de los pili tipo IV (Déziel *et al.*, 2001) y posteriormente con la resistencia a antibióticos (Drenkard & Ausubel, 2002). En el caso particular de los pacientes con fibrosis quística, los aislados de *P. aeruginosa* muestran una variación fenotípica muy significativa dando lugar a un amplio espectro de variantes como por ejemplo colonias mucosas y altamente adherentes, ausencia de movilidad, resistencia a la fagocitosis por macrófagos, resistencia a múltiples antibióticos, etc. (Deretic *et al.*, 1994; Häuβler *et al.*, 2003; Kresse *et al.*, 2003; Mahenthiralingam *et al.*, 1994; Oliver *et al.*, 2000).

Recientemente se ha demostrado que en varias especies de *Pseudomonas* los variantes fenotípicos juegan un papel importante en la colonización de la raíz. De forma natural, se ha observado que distintas *Pseudomonas* muestran variación fenotípica durante la colonización de la rizosfera (Achouak *et al.*, 2004; Sánchez-Contreras *et al.*, 2002). En *P. brassicacearum* NFM421 aparecen dos tipos de colonias morfológicamente diferentes, denominados fase I (que es similar a la cepa silvestre) y fase II (Achouak *et al.*, 2004). Las células fase II de *P. brassicacearum* presentan una sobreproducción de flagelina que da lugar a una mayor movilidad en comparación con las de fase I, al igual que los variantes fenotípicos descritos en *P. fluorescens* F113 (Achouak *et al.*, 2004; Sánchez-Contreras *et al.*, 2002). En estos variantes de *P. fluorescens* F113, la mayor producción de flagelina repercute en la síntesis de unos flagelos mucho más largos que los de la cepa silvestre. Tanto los variantes de fase II de *P. brassicacearum* NFM421 como los variantes de fase de *P. fluorescens* F113 obtenidos tras la colonización de la rizosfera se encuentran mayoritariamente en el ápice de la raíz principal y en las raíces secundarias, mientras que las colonias silvestres (fase I en NFM421) se localizan principalmente en las partes basales de la raíz (Achouak *et al.*, 2004; Sánchez-Contreras, 2001), de

ahí que la variación fenotípica en estas *Pseudomonas* se sugiera como una estrategia para aumentar la eficacia de colonización de zonas más inaccesibles. Del mismo modo, en *P. putida* DOT-T1E la expresión del gen *flhB*, que codifica una proteína que forma parte del aparato de exportación flagelar, está regulada por variación de fase como respuesta a cambios ambientales (Segura *et al.*, 2004).

En *P. florescens* WCS365 también se ha sugerido de forma indirecta la importancia de la variación fenotípica en la colonización. Se ha visto que un mutante en la recombinasa específica de sitio *sss* presenta una reducción en la competitividad, probablemente porque las células quedan bloqueadas en un fenotipo menos competitivo (Dekkers *et al.*, 1998b). Este nexo de unión entre la variación de fase, la colonización de la rizosfera y las recombinasas específicas de sitio también se ha estudiado en *P. fluorescens* F113 (Martínez-Granero *et al.*, 2005; Sánchez-Contreras *et al.*, 2002). Se ha visto que tanto Sss como XerD son responsables de la variación fenotípica en F113 y que este fenómeno está estrechamente ligado con el sistema de dos componentes *gacA*/*gacS* y la movilidad. Todos estos variantes fenotípicos muestran un fenotipo común, una mayor movilidad que la cepa silvestre. Pero sólo durante la colonización de la rizosfera se seleccionan variantes con fenotipos hipermóviles que presentan una mayor competitividad, indicando que en la rizosfera existe una presión selectiva hacia la aparición de variantes fenotípicos más móviles (Martínez-Granero *et al.*, 2006).

No sólo en *P. fluorescens* F113 se ha relacionado la variación fenotípica con el sistema *gacA*/*gacS*. En *Pseudomonas* sp. PCL1171, PCL1563, PCL1572, PCL1157, PCL1182 y PCL1184 también se ha observado esta relación (van den Broek *et al.*, 2003). Con respecto a especies de *Pseudomonas* patogénicas, se ha visto que en *P. tolaasii* RecA provoca una duplicación en *pheN*, que es homólogo a *gacS*, dando lugar a un cambio en la morfología de la colonia y en la patogenicidad (Han *et al.*, 1997; Sinha *et al.*, 2000).

Todos estos ejemplos muestran que la variación de fase (fenotípica) en las especies de *Pseudomonas* está regulando un gran número de factores, sugiriendo que este fenómeno es un mecanismo relevante en la ecología y el comportamiento de estas especies.

4. MOVILIDAD Y ADHERENCIA

Las estructuras que permiten a los organismos procariotas desplazarse o adherirse a superficies son los flagelos y las fimbrias o pili.

La distribución de los flagelos varía según las estirpes y puede ser monótrica polar como en *P. aeruginosa* (Gilardi, 1985) o perítrica (lateral) como *E. coli* y *Salmonella* (Macnab, 1996). Sin embargo, se han encontrado bacterias que presentan varios flagelos polares como es el caso de *Helicobacter pylori* y *P. putida* (Geis *et al.*, 1993; Harwood *et al.*, 1989) y otras, como algunas especies de *Aeromonas*, *Azospirillum*, *Rhodospirillum* y *Vibrio*, que presentan los dos tipos de flagelos (Allen & Baumann, 1971; Hall & Krieg, 1984; McClain *et al.*, 2002; Shimada *et al.*, 1985). En el caso de las bacterias que presentan los dos tipos de flagelos, normalmente, los flagelos polares se producen continuamente y los laterales sólo se sintetizan cuando se encuentran sobre superficies sólidas (McCarter, 2004; McClain *et al.*, 2002). Los flagelos polares permiten el movimiento conocido como *swimming* que se realiza en medio líquido y los perítricos el de *swarming* que permite un movimiento sobre superficies sólidas o en ambientes viscosos (Harshey, 1994). Aunque esto último parece que es un poco flexible ya que en estudios recientes se ha visto que *P. aeruginosa*, que presenta flagelación polar, puede realizar *swarming* en ciertas condiciones aumentando incluso el número de flagelos (Köhler *et al.*, 2000).

Dentro de las estructuras denominadas fimbrias o pili, las más estudiadas son los pili tipo IV ya que intervienen en un gran número de actividades biológicas

en la bacteria. Estas fimbrias polares contribuyen a la unión de la bacteria a superficies tanto abióticas como bióticas, interviniendo así en los primeros pasos de colonización e infección (Hahn, 1997; Roine *et al.*, 1998). Como ya se ha comentado anteriormente, estas estructuras se relacionan con un tipo de movimiento conocido como *twitching* que permite a la bacteria moverse entre dos superficies (Darzins & Russell, 1997), aunque más recientemente se ha observado que en *P. aeruginosa* también son necesarias para el *swarming* (Köhler *et al.*, 2000). Relacionado con este papel en adhesión y *twitching*, se ha visto que los pili tipo IV son importantes para la formación de biopelículas (Chiang & Burrows, 2003; Drenkard & Ausubel, 2002; O'Toole & Kolter, 1998). Pero los pili tipo IV no sólo están implicados en movilidad y adhesión sino que también juegan un papel importante en la adquisición de ADN del ambiente (Graupner *et al.*, 2000) y en la sensibilidad a bacteriófagos (Yang *et al.*, 2004). Muchas de estas funciones son dependientes de la habilidad que tienen estas estructuras para extenderse y contraerse.

La formación del flagelo supone un gran coste energético que implica la expresión de más de 50 genes diferentes. Los productos de estos genes incluyen proteínas reguladoras, componentes estructurales, proteínas encargadas de generar la fuerza del motor de rotación del flagelo y proteínas que conforman la maquinaria que controla el movimiento quimiotáctico del flagelo. Para asegurarse la máxima eficiencia y precisión durante la biosíntesis del flagelo, la bacteria emplea una compleja organización jerarquizada para controlar la expresión ordenada de todos los componentes del flagelo. Esta organización se conoce relativamente bien para algunas especies como *E. coli* y *S. typhimurium* (Chilcott & Hughes, 2000; Macnab, 1996; Macnab, 2003).

Los genes implicados en la formación del flagelo en *E. coli* y *S. typhimurium* se clasifican en tres clases según su expresión y el momento o factores que intervienen en ella. La clase I está formada por los genes *flhC* y *flhD* que

constituyen el operón regulador principal o "master operon". La transcripción de este operón es dependiente del factor σ^{70} (Kutsukake, 1997) y está regulado por la proteína receptora de AMPc (adenosin monofosfato cíclico) CRP (Soutourina *et al.*, 1999). Dentro de la regulación de *flhDC*, se ha encontrado un sistema de dos componentes denominado EnvZ/OmpR que es capaz de reprimir la expresión de este operón tanto en *E. coli* (Shin & Park, 1995) y como en *Xenorhabdus nematophila* (Kim *et al.*, 2003; Park & Forst, 2006), mientras que su implicación en *S. typhimurium* no está tan clara (Kutsukake, 1997). Más recientemente en *E. coli*, se ha visto que este "master operon" está también regulado positivamente por otro sistema de dos componentes, que responde a *quorum sensing*, denominado QseB/QseC (González Barrios *et al.*, 2006; Sperandio *et al.*, 2002) y por la proteína de unión a ARN CsrA (Wei *et al.*, 2001). La función de este regulador principal es activar la expresión de los genes de clase II (Liu & Matsumura, 1994), pero además está implicado en otras funciones en distintas especies como por ejemplo en *E. coli* que está también involucrado en la división celular (Prüβ & Matsumura, 1996), en *Xenorhabdus nematophila* que regula factores de virulencia y producción de fosfolipasa (Givaudan & Lanois, 2000) y en *Proteus mirabilis* que regula la expresión de la toxina hemolisina (Fraser *et al.*, 2002). Los genes de clase II codifican proteínas del cuerpo basal, del gancho del flagelo y el factor sigma FliA (σ^{28}). El factor σ^{28} a su vez está implicado en la transcripción de los genes de clase III que son necesarios para el ensamblaje del filamento flagelar, del motor del flagelo y para la quimiotaxis. La actividad de FliA está bloqueada a nivel post-traduccional por la acción del factor anti-sigma FlgM (Chadsey *et al.*, 1998), de forma que sólo estará activa cuando FlgM se empiece a secretar a través de la estructura completa del cuerpo basal-gancho (Hughes *et al.*, 1993).

En las especies de *Pseudomonas* los genes responsables de la formación del flagelo están menos estudiados. Encontramos aspectos comunes y diferencias con lo que hay descrito para enterobacterias. Se sabe también que la organización es jerárquica, aunque en este caso, no se han encontrado indicios de la existencia de un

operón regulador principal (Dasgupta *et al.*, 2003). Es en *P. aeruginosa* donde se ha profundizado más en el estudio de la síntesis y regulación del flagelo ya que se ha demostrado que los flagelos juegan un papel importante en la virulencia de este patógeno oportunista (Arora *et al.*, 1998; Feldman *et al.*, 1998).

Entre los genes que se encuentran en *P. aeruginosa* pero no en enterobacterias están varios reguladores como el factor transcripcional FleQ (Arora *et al.*, 1997) y el sistema de dos componentes FleSR (Ritchings *et al.*, 1995). Este activador de la familia NtrC/NifA denominado FleQ o AdnA (Casaz *et al.*, 2001) también ha sido identificado en otras pseudomonas como factor importante en la síntesis del flagelo. Se sabe que FleQ regula la producción de la flagelina FliC y su exportación en *P. fluorescens* F113 (Capdevila *et al.*, 2004) y de otras proteínas estructurales del flagelo y del aparato quimiotáctico en *P. fluorescens* Pf0-1 (Robleto *et al.*, 2003). En las pseudomonas la flagelina muestra una heterogeneidad o polimorfismo que no se encuentra en enterobacterias. Este polimorfismo viene dado por la presencia de dos tipos de flagelina (por ejemplo, flagelina de tipo a en *P. aeruginosa* PAK y flagelina de tipo b en *P. aeruginosa* PAO1) y por la glicosilación de las mismas (Arora *et al.*, 2001; Spangenberg *et al.*, 1996). No sólo la flagelina presenta este polimorfismo, la proteína del extremo del filamento flagelar, FliD, también se puede dividir en dos tipos (Arora *et al.*, 2000). Dentro de las diferencias también se ha encontrado un factor sigma, el factor σ^{54} o RpoN, que junto con FleQ intervienen en la expresión de gran parte de los genes que conforman el flagelo (Dasgupta *et al.*, 2003). Sin embargo, al igual que en enterobacterias, se ha descrito un factor σ^{28} codificado por *fliA* que se regula negativamente por FlgM y que activa la expresión de la flagelina (Frisk *et al.*, 2002; Starnbach & Lory, 1992).

Mediante la comparación de la expresión de genes implicados en la síntesis del flagelo tanto en la cepa silvestre como en los mutantes *rpoN*, *fleQ*, *fleR* y *fliA*, se llegó a la conclusión de que *P. aeruginosa* emplea una regulación jerárquica a

cuatro niveles (Dasgupta *et al.*, 2003) (Fig. 1.5), al igual que otras bacterias monoflageladas como *Vibrio cholerae*, *Vibrio parahaemolyticus* y *Caulobacter crecentus* (Gober & England, 2000; Kim & McCarter, 2000; Prouty *et al.*, 2001).

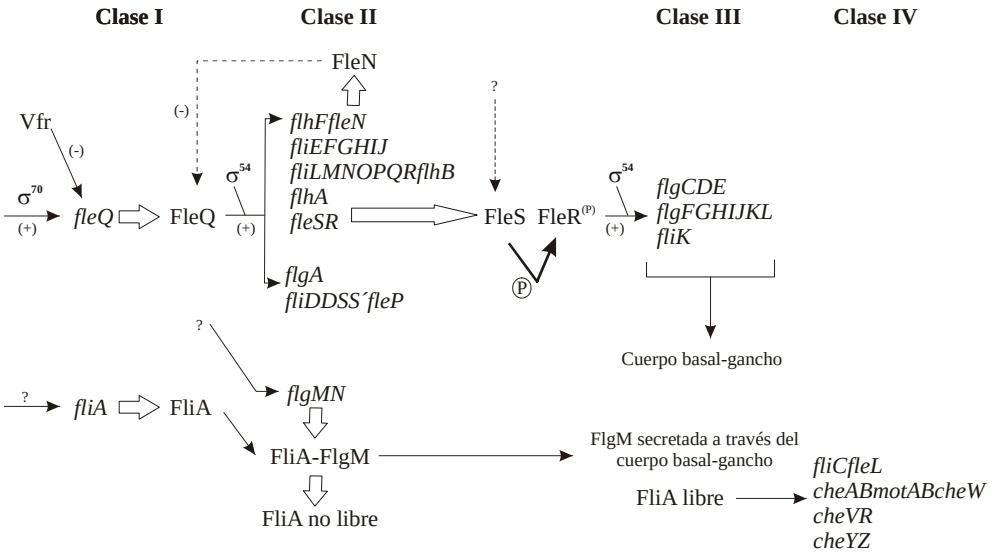

Figura 1.5. Transcripción jerárquica de varios genes flagelares en *P. aeruginosa*. (-) y (+) indican regulación negativa y positiva, respectivamente. ? indica factor(es) desconocido(s), (P) indica fosforilación. Adaptación de Dasgupta y col. (2003).

Basándose en lo que se conoce hasta ahora de la biogénesis flagelar en *P. aeruginosa*, se puede considerar a FleQ como el regulador principal ya que regula, directa o indirectamente, la expresión de la mayoría de genes flagelares con la excepción de *fliA* (Dasgupta *et al.*, 2003). Tanto *fleQ* como *fliA* se agrupan en la clase I ya que su expresión no está influenciada por ninguno de los reguladores flagelares conocidos. Sin embargo, hay evidencias que indican que σ^{70} activa la transcripción de *fleQ* y que Vfr, el homólogo de CRP en *E. coli*, la reprime (Dasgupta *et al.*, 2002). Estudios recientes también demuestran que el factor sigma AlgT (AlgU o σ^{22}), que regula la producción de alginato, reprime la expresión de

fleQ en *P. aeruginosa* y en consecuencia la síntesis flagelar (Tart *et al.*, 2005). De hecho, esta regulación inversa entre el control del flagelo y la biosíntesis de exopolisacárido se ha observado en otras bacterias como *E. coli* y *V. cholerae* (Prigent-Combaret *et al.*, 1999; Watnick *et al.*, 2001).

Los genes de clase II, que codifican componentes estructurales del cuerpo basal, anillo MS, motor, aparato de exportación flagelar y extremo del filamento, están regulados por FleQ y σ^{54}. Dentro de los genes de clase II también hay proteínas reguladoras como FlhF, FleN, FleS y FleR (Dasgupta *et al.*, 2003). En los sistemas flagelares perítricos de *E. coli* y *S. typhimurium* no se han encontrado homólogos para estas proteínas reguladoras (Dasgupta *et al.*, 2004). Como FlhF interviene en el emplazamiento del flagelo polar en *P. putida* (Pandza *et al.*, 2000) y debido a que presenta una gran homología con la de *P. aeruginosa* es probable que también determine la localización flagelar en esta última (Dasgupta *et al.*, 2003). Por otro lado, FleN juega un papel crucial en el mantenimiento del número de flagelos, mediante la regulación negativa de FleQ a nivel post-traduccional (Dasgupta & Ramphal, 2001).

La activación de FleR a través de FleS parece que es necesaria para la transición de clase II a clase III, marcando un nuevo punto en el proceso de síntesis del flagelo en *P. aeruginosa*. Sin embargo, la señal que detecta FleS no se conoce todavía. Dada su posible localización en el citoplasma (Ritchings *et al.*, 1995), puede que esté detectando la finalización de una estructura o el exceso de un intermediario estructural. Aparte de FleR activo también es necesario RpoN para la expresión de los genes de clase III, que codifican proteínas necesarias para la finalización de la estructura del cuerpo basal-gancho (Dasgupta *et al.*, 2003).

Una vez que la estructura del cuerpo basal-gancho está completa, FlgM se secreta a través de él por un mecanismo similar al descrito para *S. typhimurium*

(Karlinsey *et al.*, 2000) dejando libre a FliA para expresar los genes de clase IV (filamento, rotación del motor y quimiotaxis) (Dasgupta *et al.*, 2003).

5. METABOLISMO SECUNDARIO EN PSEUDOMONAS

Las *Pseudomonas* pueden producir una mezcla compleja de metabolitos secundarios como por ejemplo sideróforos (pioverdina, pioquelina, pseudobactina y ferribactina) y compuestos antibióticos y antifúngicos [cianhídrico (HCN), floroglucinol (DAPG o Phl), fenacinas (PCA y PCN), pioluteorina, pirrolnitrina, lipopéptidos cíclicos (visconamida, tensina, anfisina, etc) y exo-enzimas] (Haas & Défago, 2005; Koch *et al.*, 2002; Lugtenberg & Bloemberg, 2004; Nielsen *et al.*, 2002; Raaijmakers *et al*., 2006).

Uno de los metabolitos cuya actividad ha sido mejor estudiada en *Pseudomonas*, especialmente en *P. fluorescens* CHA0, es el 2,4-diacetilfloroglucinol (DAPG o Phl). El operón de biosíntesis de DAPG *phlACBD* de *P. fluorescens* Q2-87, F113 y CHA0 está reprimido por la proteína PhlF, miembro de la familia TetR (Bangera & Thomashow, 1999; Delany *et al.*, 2000; Schnider-Keel *et al.*, 2000). PhlF reprime la síntesis uniéndose en forma de dímero a la región promotora de *phlA*. El propio DAPG controla positivamente su biosíntesis ya que la adición de éste previene la unión de PhlF (Schnider-Keel *et al.*, 2000). Sin embargo, otros compuestos aromáticos como la pioluteorina (también producida por CHA0), el ácido salicílico y el ácido fusárico (toxina producida por el hongo *F. oxysporum*) antagonizan el efecto des-represor del DAPG, dando lugar a una represión del operón *phlACBD* a través de PhlF (Notz *et al.*, 2002; Schnider-Keel *et al.*, 2000). La producción de DAPG también puede estar influenciada por factores abióticos como el Zn^{2+}, el Cu^{2+}, el Fe^{3+}, la tensión de oxígeno y las fuentes de carbono (Duffy & Défago, 1999). En *P. fluorescens* CHA0 se ha identificado un segundo regulador tipo TetR denominado PhlH que parece que actúa como activador o anti-represor del operón *phl* (Schnider-Keel *et al.*, 2000).

Además del DAPG, *P. fluorescens* CHA0 produce pioluteorina, pirrolnitrina y cianhídrico (Haas & Keel, 2003). La importancia relativa de todos estos compuestos en el control de enfermedades depende de la planta, sugiriendo que hay factores específicos de la planta que podrían estar implicados en la producción de estos metabolitos. Por ejemplo, la producción de pioluteorina por *P. fluorescens* está asociada con la supresión del "damping off" en berro pero no en pepino (Kraus & Loper, 1992; Maurhofer *et al.*, 1994b).

En *P. fluorescens* Pf-5, los genes *pltLABCDEFG*, implicados en la síntesis de pioluteorina, se regulan positivamente por el regulador tipo LysR PltR (Nowak-Thompson *et al.*, 1999). Al igual que la autorregulación positiva de la producción de DAPG, la síntesis de pioluteorina es probable que presente un circuito de autorregulación similar ya que la adición de pioluteorina a cultivos de *P. fluorescens* Pf-5 y CHA0 provoca un aumento en la expresión de los genes *plt* (Brodhagen *et al.*, 2004; Haas & Keel, 2003). Además todo apunta a que hay un mecanismo de control mutuo que permite a estas dos pseudomonas alcanzar una producción de antibióticos equilibrada, ya que en estas dos bacterias el DAPG reprime la síntesis de pioluteorina y viceversa (Haas & Keel, 2003; Schnider-Keel *et al.*, 2000).

La respiración microbiana y la captación de oxígeno por parte de las raíces generan un descenso de oxígeno en la superficie radicular. Muchos microorganismos del suelo son capaces de adaptarse rápidamente a estas concentraciones de oxígeno cambiantes. En concreto en pseudomonas el encargado de estos cambios es el regulador transcripcional ANR, proteína tipo FeS homóloga al regulador FNR de enterobacterias, que es capaz de cambiar entre un estado activo a bajas concentraciones de oxígeno y un estado inactivo a altas concentraciones (Ye *et al.*, 1995). Aparte de regular rutas de desnitrificación importantes para crecer en condiciones de bajo oxígeno, ANR regula positivamente la expresión del operón

hcnABC encargado de la síntesis de HCN en *P. fluorescens* CHA0 (Laville *et al.*, 1998). Algunas observaciones hechas en *P. aeruginosa*, que también presenta su propio operón *hcnABC*, sugieren que hay más elementos reguladores en la síntesis de cianhídrico. Se ha visto que los reguladores LasR y RhlR, implicados en *quorum sensing*, aumentan la transcripción de los genes *hcnABC* en *P. aeruginosa* (Pessi & Haas, 2000). Esta regulación, sin embargo, está ausente en algunas pseudomonas como por ejemplo *P. fluorescens* CHA0 (Haas *et al.*, 2002).

La activación mediada por densidad celular (*quorum sensing*) también regula la síntesis de fenacina en varias *Pseudomonas*. El operón *phz*, responsable de la síntesis de fenacina, de *P. aureofaciens* 30-84, *P. fluorescens* 2-79 y *P. chlororaphis* PCL1391 está controlado positivamente por el factor transcripcional PhzR (Chin-A-Woeng *et al.*, 2001; Khan *et al.*, 2005; Pierson *et al.*, 1994). Este activador es un miembro de la familia LuxR, y como tal, requiere de una señal como por ejemplo acil-homoserina lactona para su activación. La producción de dicha señal en estas pseudomonas está catalizada por PhzI, una acil-homoserina lactona sintasa de la familia LuxI (Chin-A-Woeng *et al.*, 2001; Khan *et al.*, 2005; Wood & Pierson, 1996). En *P. aeruginosa* PAO1, la síntesis de fenacina también se encuentra bajo el control del *quorum sensing*, pero en este caso es más compleja e implica a tres reguladores tipo LuxR, los activadores LasR y RhlR, al igual que en la síntesis de cianhídrico, y el represor QscR (Ledgham *et al.*, 2003; Pessi & Haas, 2000).

El efecto negativo del ácido fusárico parece que no sólo está restringido a la producción de DAPG ya que también se ha visto que tiene una influencia negativa en la producción de fenacina en *P. chlororaphis* PCL1391 y *P. fluorescens* WCS365 (Lugtenberg & Bloemberg, 2004; van Rij *et al.*, 2004; van Rij *et al.*, 2005). Parece que es un mecanismo general de defensa del hongo frente al ataque por estas pseudomonas.

Aunque el hierro es uno de los metales más abundantes, la baja solubilidad en condiciones aeróbicas a pH neutro hace que la biodisponibilidad de este elemento sea baja. Muchas bacterias han solucionado este problema sintetizando moléculas de bajo peso molecular, denominadas sideróforos, que son capaces de solubilizar el hierro y hacerlo disponible para la célula (Neilands, 1995). Dentro de las *Pseudomonas* los sideróforos más importantes son las pioverdinas o pseudobactinas, que son producidos por las pseudomonas fluorescentes (Budzikiewicz, 1993; Meyer, 2000). Aunque también existen otros tipos de sideróforos como la pioquelina, pseudomonina, ácido piridina-2,6-ditiocarboxílico, quinolobactina, etc., producidos tanto por pseudomonas fluorescentes como no-fluorescentes (Cox *et al.*, 1981; Duffy & Défago, 2000; Mercado-Blanco *et al.*, 2001; Mossialos *et al.*, 2000; Stolworthy *et al.*, 2001).

Una vez se ha formado el complejo sideróforo-Fe, éstos se unen a receptores de la membrana externa (bacterias Gram negativas), que pueden ser específicos de un complejo sideróforo-Fe o menos específicos, pudiendo incluso captar otras fuentes de hierro como el citrato férrico, grupos hemo, lactoferrina y transferrina (Blanton *et al.*, 1990; Cornelissen, 2003; Massé & Arguin, 2005; Ochsner *et al.*, 2000). El transporte de estos complejos a través de los receptores de membrana externa requiere el sistema TonB-ExbB-ExbD, que utiliza la energía procedente del gradiente electroquímico de la membrana interna para llevarlo a cabo (Higgs *et al.*, 1998; Koebnik, 2005). Una vez en el periplasma, los complejos sideróforo-Fe son transportados a través de la membrana interna por la acción de sistemas transportadores tipo ABC (Köster, 2001).

La captación de hierro está fuertemente regulada para evitar una acumulación excesiva que puede llegar a ser tóxica para la célula (Braun, 1997; Touati, 2000). En *Pseudomonas*, esta captación está regulada por la proteína represora Fur (**f**erric **u**ptake **r**egulator) y su cofactor, Fe^{2+}, que controlan la transcripción de los factores sigma extracitoplasmáticos PvdS y FpvI (Redly &

Poole, 2003; Visca *et al.*, 2002). PvdS y FpvI, a su vez, son necesarios para la transcripción de los genes de biosíntesis de pioverdina y de sus receptores, respectivamente (Leoni *et al.*, 2000; Redly & Poole, 2003; Sexton *et al.*, 1996; Visca *et al.*, 2002; Wilson *et al.*, 2001). Dentro de la síntesis de pioverdina también podemos encontrar otros reguladores, que no están relacionados aparentemente con los niveles de hierro en la célula, como el factor sigma RpoS (σ^s) que afecta negativamente la producción de este sideróforo en *P. aeruginosa* (Suh *et al.*, 1999) o el sistema LasI/LasR que, al contrario de RpoS, es necesario para su síntesis (Stintzi *et al.*, 1998). Fur también puede actuar como regulador positivo, aumentando la producción de factores que mitigan la toxicidad del hierro y de proteínas no esenciales que contienen hierro (Dubrac & Touati, 2000; Massé & Gottesman, 2002; Massé & Arguin, 2005).

Para las *Pseudomonas* el hierro supone un punto muy importante en la colonización y competencia por los nichos donde habitan. En *P. aeruginosa* se ha encontrado que la producción de pioverdina tiene una gran importancia en la colonización de sus huéspedes (Handfield *et al.*, 2000; Meyer *et al.*, 1996; Takase *et al.*, 2000). De hecho, algunas cepas de *P. putida*, *P. fluorescens* y *P. aeruginosa* tienen la capacidad de utilizar un amplio espectro de pioverdinas heterólogas procedentes de bacterias competidoras (Koster *et al.*, 1995; Meyer *et al.*, 1999; Mirleau *et al.*, 2000; Ongena *et al.*, 2001; Raaijmakers *et al.*, 1995). Además, parece que estos sideróforos también pueden contribuir en ciertas situaciones al efecto biocontrolador de muchas *Pseudomonas* ya sea por la competición por el hierro, por la posible acción antibiótica de sus cadenas peptídicas o de los propios complejos sideróforo-metal o por la inducción de una resistencia sistémica en la planta (Cornelis & Matthijs, 2002; Haas & Défago, 2005; Keel *et al.*, 1989; Kloepper *et al.*, 1980; Leeman *et al.*, 1996; Loper & Buyer, 1991; Maurhofer *et al.*, 1994a; Scher & Baker, 1982).

5.1. Sistema de dos componentes GacA/GacS

En las bacterias la transducción de señales ambientales a menudo está mediada por pares de proteínas formados por una proteína sensora y una reguladora, conocidos como sistema regulador de dos componentes. Estos sistemas reguladores ayudan a estos organismos a adaptarse a las diferentes condiciones en respuesta a señales ambientales, que pueden ser tanto abióticas (pH, temperatura, osmolaridad, etc) como bióticas (producidas por el huésped o por las propias bacterias).

En estos sistemas, la proteína sensora posee un dominio transmembrana que es responsable de la captación del estímulo y otro dominio con actividad quinasa. Cuando el sensor recibe el estímulo es capaz de autofosforilarse y transferir dicho grupo fosfato al componente regulador, activándolo. Este regulador es una proteína citoplasmática, que una vez activa, promueve cambios en la expresión de los genes diana.

El sistema de dos componentes GacA/GacS es un sistema que está presente en una gran variedad de bacterias; se han identificado unos 20 homólogos a GacS/GacA, denominados BarA/UvrY en *E. coli*, BarA/SirA en *Salmonella enterica*, ExpS/ExpA en *Erwinia carotovora*, BarA/VarA en *Vibrio cholerae* y GacS(LemA, PheN)/GacA en *Pseudomonas* (Heeb & Haas, 2001). Normalmente los genes que conforman estos sistemas de dos componentes están localizados en el genoma de forma adyacente, pero en el caso del sistema Gac esto no es así. Sin embargo, se ha observado que la organización del locus *gacA* está bastante conservada entre distintas especies. Este regulador está seguido por el homólogo del gen *uvrC* de *E. coli*, que está implicado en la reparación del ADN en respuesta a la radiación ultravioleta. De hecho, mutaciones polares en el gen *gacA* provocan una disminución drástica en la tolerancia al ultravioleta (Laville *et al.*, 1992; Reimmann *et al.*, 1997).

El sistema GacA/GacS controla la síntesis de metabolitos secundarios y exo-enzimas implicados en la acción biocontroladora de pseudomonas beneficiosas y en la virulencia de bacterias patógenas (Aarons *et al.*, 2000; Gaffney *et al.*, 1994; Haas & Keel, 2003; Heeb & Haas, 2001; Koch *et al.*, 2002; Laville *et al.*, 1992; Tomenius *et al.*, 2006).

Con respecto a la señal que activa el sistema GacA/GacS no se conoce mucho. Se sabe que los metabolitos que regula este sistema se expresan mayoritariamente cuando el cultivo está en la transición de fase exponencial a estacionaria (Blumer *et al.*, 1999; Heeb & Haas, 2001; Reimmann *et al.*, 1997), indicando que puede haber implicado un mecanismo del tipo *quorum sensing* regulando el sistema Gac. De hecho, la cepa biocontroladora *P. fluorescens* CHA0 sintetiza una señal extracelular que activa la producción de los metabolitos regulados por Gac (Heeb *et al.*, 2002; Zuber *et al.*, 2003). Esta señal tiene un peso molecular bajo y no es una acil-homoserina lactona (AHL). Aunque todavía no se sabe cuál es la señal exacta que dispara este sistema en *P. fluorescens* CHA0, recientemente se ha visto que el gen *thiC*, implicado en la ruta de biosíntesis de tiamina, juega un papel crucial en la síntesis de la señal (Dubuis *et al.*, 2006). Esta tiamina quizás sea un cofactor esencial para alguna enzima implicada en la síntesis de la señal o puede que esté afectando indirectamente su producción.

Como ya se ha mencionado antes, el denominador común del sistema GacA/GacS es la regulación de la producción de metabolitos secundarios y exo-enzimas. Dentro de las bacterias beneficiosas de plantas (*P. fluorescens* CHA0, Pf-5, BL915 y F113, *Pseudomonas* sp. PCL1171 y *P. aureofaciens* 30-84 entre otras), regula positivamente la producción de metabolitos importantes para la protección frente a agentes fitopatógenos como el DAPG, pioluteorina, pirrolnitrina, HCN, fenacinas y lipopéptidos (Aarons *et al.*, 2000; Bull *et al.*, 2001; Chancey *et al.*, 2002; Duffy & Défago, 2000; Gaffney *et al.*, 1994; Koch *et al.*, 2002; Kraus & Loper, 1992; Laville *et al.*, 1992; Natsch *et al.*, 1994; Pfender *et al.*, 1993; Sánchez-

Contreras *et al.*, 2002; van den Broek *et al.*, 2003). En estas *Pseudomonas*, el sistema GacA/GacS regula también positivamente la expresión de genes que codifican enzimas que se secretan como por ejemplo exoproteasa, fosfolipasa C y quitinasa (Aarons *et al.*, 2000; Chabeaud *et al.*, 2001; Gaffney *et al.*, 1994; Heeb *et al.*, 2002; Martínez-Granero *et al.*, 2005; Sacherer *et al.*, 1994; Sánchez-Contreras *et al.*, 2002; van den Broek *et al.*, 2003). Sin embargo, GacA/GacS también puede ejercer un control negativo sobre ciertos metabolitos como los sideróforos. En *P. fluorescens* CHA0, los mutantes *gacA* y *gacS* muestran una mayor producción de pioquelina y de un compuesto fluorescente que probablemente es pioverdina (Duffy & Défago, 2000; Schmidli-Sacherer *et al.*, 1997). Este fenotipo se repite también para *P. fluorescens* F113 ya que el variante F, que presenta un aumento en la expresión del gen *pvdA* y en consecuencia de la pioverdina, recupera los niveles silvestres cuando se introduce el gen *gacA* en *trans* (Sánchez-Contreras *et al.*, 2002). Estudios posteriores con variantes procedentes de la sobreexpresión de dos recombinasas específicas de sitio en *P. fluorescens* F113 corroboraron que el sistema Gac regula negativamente la producción de pioverdina en esta cepa (Martínez-Granero *et al.*, 2005).

Este sistema GacA/GacS también es esencial para la virulencia de muchas bacterias patógenas. En el patógeno oportunista *P. aeruginosa*, está implicado en la regulación de los sistemas de *quorum sensing las* y *rhl* que a su vez regulan la producción de factores de virulencia extracelulares como HCN, piocianina y elastasa (Kay *et al.*, 2006; Pessi *et al.*, 2001; Reimmann *et al.*, 1997). Además, los mutantes *gac* de *P. aeruginosa* son menos virulentos que la cepa silvestre en varios modelos (Jander *et al.*, 2000; Rahme *et al.*, 1995; Tan *et al.*, 1999). Un mutante *gacA* de *P. syringae* pv. *tomato* DC3000 presenta unos niveles de expresión reducidos de *rpoN* y *hrpRS*, que son necesarios para la expresión de genes que forman parte del sistema de secreción tipo III (TTSS) (Chatterjee *et al.*, 2003). Este mutante *gacA* de *P. syringae* también está afectado en la producción de otros factores de virulencia como la fitotoxina coronatina. En otros patógenos foliares

como *P. syringae* pv. *syringae* B728a, *P. viridiflava* y *P. marginalis* y en el patógeno del champiñón *P. tolaasii*, este sistema Gac también juega un papel crucial en patogenicidad (Han *et al.*, 1997; Hrabak & Willis, 1992; Liao *et al.*, 1996; Liao *et al.*, 1997; Rich *et al.*, 1994; Willis *et al.*, 2001). En patógenos no relacionados con *Pseudomonas* también se ha observado esta relación entre el sistema Gac y virulencia. En *Salmonella enterica* serovar *typhimurium*, el sistema BarA/SirA regula positivamente el TTSS y otros factores implicados en invasión y virulencia (Goodier & Ahmer, 2001) y en *Vibrio cholerae* (BarA/VarA) es responsable de la producción de la toxina colérica (Wong *et al.*, 1998).

Otro factor que de alguna forma está también vinculado al biocontrol y a la virulencia, y que está regulado por el sistema Gac, es la movilidad. Como ya se ha mencionado en el párrafo anterior, este sistema de dos componentes regula algunos factores importantes para la virulencia de *P. aeruginosa*. Así, no es sorprendente que también regule la movilidad de este patógeno oportunista ya que la movilidad se ha descrito como un factor importante para la colonización de los nichos y la virulencia. En concreto, parece que este sistema regula negativamente la movilidad tipo *swarming* y *swimming* ya que un mutante *rsmA*, que como se indica más adelante es una proteína de la cascada Gac, muestra un fenotipo inmóvil (en experimentos de movimiento tipo *swarming*) (Heurlier *et al.*, 2004) o una movilidad reducida (en experimentos de movimiento tipo *swimming*) (Burrowes *et al.*, 2006). De hecho, en un trabajo posterior del mismo grupo se observa que un mutante *gacA*, que equivale a la sobreexpresión de *rsmA*, es más móvil (en experimentos de análisis del movimiento tipo *swarming*) que la cepa silvestre y que además presenta una mayor expresión de la flagelina (FliC) y de la proteína extremo del filamento flagelar (FliD) (Kay *et al.*, 2006). Al contrario que en *P. aeruginosa*, las mutaciones en el sistema Gac de las *Pseudomonas* fitopatógenas *P. syringae* B728a y DC3000 provocan una reducción en la movilidad tipo *swarming* (Chatterjee *et al.*, 2003; Kinscherf & Willis, 1999; Quiñones *et al.*, 2005). Al igual que en las *Pseudomonas* patógenas, dentro de las bacterias patógenas como *Salmonella* y *E. coli* podemos

encontrar diferencias en cuanto a la regulación de la movilidad. El master operón *flhDC* en *E. coli*, responsable de la síntesis del flagelo, está sujeto a regulación post-transcripcional positiva a través de un homólogo a RsmA denominado CsrA (Wei *et al.*, 2001). Sin embargo, un mutante *sirA* (*gacA*) en *Salmonella enterica*, que equivale a la sobreexpresión de *rsmA*, muestra unos niveles de expresión de los genes responsables de la síntesis flagelar superiores a los de la cepa silvestre (Goodier & Ahmer, 2001).

No sólo en las *Pseudomonas* patógenas se ha visto que el sistema Gac regula la movilidad. Este sistema también está implicado en la movilidad de *Pseudomonas* beneficiosas como *P. fluorescens* y *P. brassicacearum*. En *P. fluorescens* CHA0, regula positivamente la movilidad tipo *swarming* ya que un mutante *gacA* es inmóvil (Kay *et al.*, 2005). Al contrario que en CHA0, GacA/GacS regula negativamente la movilidad en *P. fluorescens* F113 y *P. brassicacearum* NFM421. En *P. fluorescens* F113, los mutantes *gac* presentan una movilidad tipo *swimming* 1.5 veces superior a la cepa silvestre (Martínez-Granero *et al.*, 2006). Probablemente el *swarming* también esté reprimido a través del sistema Gac en F113 ya que el variante F, que presenta una mutación en *gacA*, muestra una mayor movilidad tanto de *swimming* como de *swarming* en comparación con la cepa silvestre (Sánchez-Contreras *et al.*, 2002). Los variantes fenotípicos de *P. brassicacearum* también muestran una mayor movilidad tanto de *swimming* como de *swarming*, a la vez que una mayor producción de flagelina (Achouak *et al.*, 2004). Aunque no se ha demostrado formalmente que estos variantes de *P. brassicacearum* sean mutantes *gac*, es bastante probable que lo sean ya que no son capaces de producir exo-enzimas (Chabeaud *et al.*, 2001) y además la morfología de las colonias de estos variantes fenotípicos es similar a la de los mutantes *gac* en *P. fluorescens* F113 y *Pseudomonas* sp. PCL1171 (Martínez-Granero *et al.*, 2005; Sánchez-Contreras *et al.*, 2002; van den Broek *et al.*, 2005b).

El hecho de que el sistema GacA/GacS o sus homólogos en miembros de géneros tan dispares como *Escherichia*, *Salmonella*, *Vibrio*, *Erwinia* y *Pseudomonas* regulen los mismos factores (metabolismo secundario, virulencia y/o movilidad), sugiere que este sistema de dos componentes controla funciones evolutivamente conservadas.

El sistema Gac también afecta a otros factores que no tienen que ver con el biocontrol, la virulencia y la movilidad. La producción de ácido indolacético (AIA), que promueve el crecimiento de la planta, está regulada negativamente por GacA/GacS en *P. chlororaphis* O6 ya que un mutante *gacS* produce unos niveles de AIA 10 veces superiores a la cepa silvestre (Kang *et al.*, 2006). Aunque parece que esta regulación es diferente en otras bacterias porque la sobreexpresión de *gacS* en *Enterobacter cloacae* CAL2 provoca un aumento en la producción de AIA (Saleh & Glick, 2001).

Hasta el momento no se han encontrado evidencias que indiquen que la expresión de todos estos factores dependientes del sistema Gac se deba a un efecto directo del regulador GacA. Sin embargo, hay resultados que demuestran que estos factores no están sujetos a un control transcripcional directo por GacA (Heeb & Haas, 2001). De hecho, se han encontrado evidencias que sugieren que la regulación de los factores de biocontrol por GacA en *P. fluorescens* CHA0 se lleva a cabo a nivel post-transcripcional (Blumer *et al.*, 1999).

Tanto en *P. fluorescens* como en el fitopatógeno *Erwinia carotovora*, se ha visto que GacA regula positivamente la expresión de un ARN regulador de pequeño tamaño que no se traduce, denominado *prrB* en F113, *rsmZ* en CHA0 y *rsmB* en *E. carotovora* (Aarons *et al.*, 2000; Heeb *et al.*, 2002; Hyytiäinen *et al.*, 2001). Más recientemente se ha encontrado que muchas bacterias no sólo presentan un único ARN regulador. En *Vibrio cholerae* y *P. fluorescens*, el sistema GacA/GacS activa la transcripción de tres ARN reguladores funcionalmente redundantes

(*rsmX/rsmY/rsmZ* en *P. fluorescens* y *csrB/csrC/csrD* en *V. cholerae*), mientras que en *P. aeruginosa*, *E. coli* y *Salmonella enterica* se han descrito dos (*rsmY/rsmZ* en *P. aeruginosa* y *csrB/csrC* en *E. coli* y *S. enterica*) (Fortune *et al.*, 2006; Kay *et al.*, 2005; Kay *et al.*, 2006; Lenz *et al.*, 2005; Weilbacher *et al.*, 2003). Estos ARN reguladores aunque no presentan una secuencia nucleotídica conservada si que muestran una estructura secundaria muy característica que se asemeja a una flor, de forma que en cada bucle aparece el motivo conservado AGG(N)A (Aarons *et al.*, 2000; Heeb & Haas, 2001; Valverde *et al.*, 2004). Además, presentan una alta afinidad por proteínas de unión a ARN que actúan principalmente como represores de la traducción, por ejemplo, CsrA (en *E. coli*, *S. enterica* y *V. cholerae*), RsmA (en *E. carotovora* y *P. aeruginosa*) y RsmA/RsmE (en *P. fluorescens*) (Fortune *et al.*, 2006; Lenz *et al.*, 2005; Liu *et al.*, 1998; Pessi *et al.*, 2001; Reimmann *et al.*, 2005; Romeo, 1998). Estas proteínas de unión a ARN son secuestradas por los ARN reguladores, dejando libre los sitios de unión a ribosoma de los ARN mensajeros (ARNm) que estaban bloqueando para que sean accesibles a la maquinaría de traducción (Valverde *et al.*, 2004). Por poner un ejemplo, en *P. fluorescens* CHA0, la sobreexpresión de *rsmZ* o la mutación de RsmA presentan un fenotipo similar, una des-represión de la síntesis de los factores responsables del biocontrol. Mientras que la sobreexpresión de RsmA da lugar a una represión de los mismos (Blumer *et al.*, 1999; Heeb *et al.*, 2002). Además, el ARN regulador *rsmB* funciona como antagonista del efecto represor de CsrA en *E. coli* (Romeo, 1998). Todos estos trabajos han dado lugar a la postulación de un modelo simplificado de la cascada de regulación del sistema GacA/GacS (Fig. 1.6).

Sin embargo este modelo no es tan sencillo como se muestra en la Figura 1.6. Por ejemplo, la proteína CsrA en *E. coli* puede actuar como represor o como activador de la traducción, dependiendo del ARNm diana al que esté unida. La unión de CsrA al ARNm de *glgCAP* impide la unión del ribosoma y desestabiliza el propio ARNm (Baker *et al.*, 2002; Liu *et al.*, 1995), mientras que la unión de CsrA

a la región 5´ del ARNm de *flhDC* aumenta su estabilidad y su traducción (Wei *et al.*, 2001).

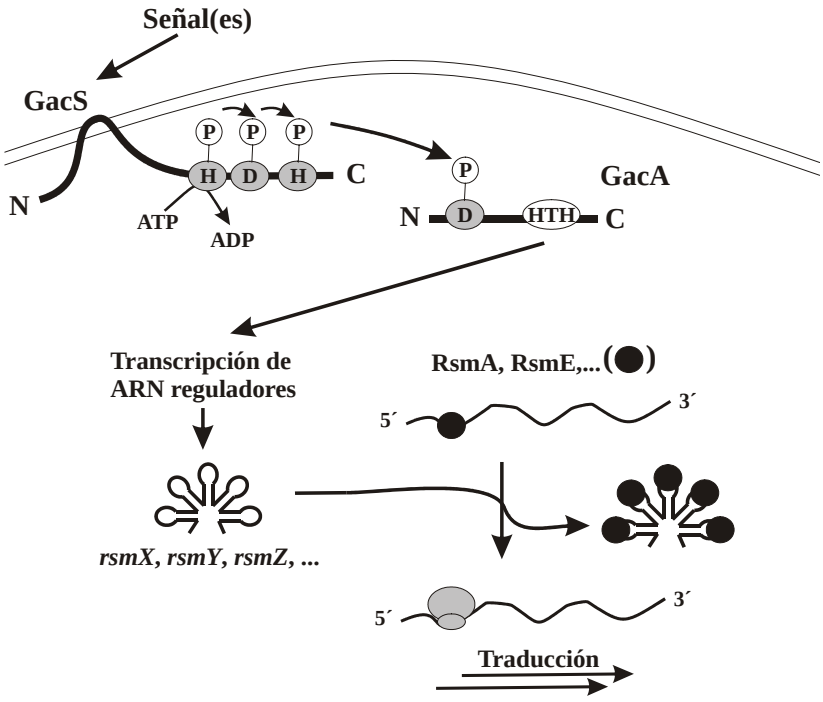

Figura 1.6. Modelo de la cascada de transducción de la señal del sistema GacA/GacS. H indica aminoácido histidina, D aminoácido aspártico y P grupo fosfato.

En *P. fluorescens* CHA0, que es la pseudomonas beneficiosa donde se han realizado más estudios en relación con este sistema de dos componentes, se ha observado que hay un mecanismo de autorregulación dentro de la cascada Gac/Rsm (Kay *et al.*, 2005; Reimmann *et al.*, 2005). En estos dos trabajos se ha visto que no sólo GacA/GacS son necesarios para la transcripción de los ARN reguladores sino que también RsmA y RsmE muestran un efecto en dicha transcripción, ya que el doble mutante *rsmA-rsmE* presenta unos niveles de transcripción de los ARN reguladores muy similares al de un mutante *gac*. Esta autorregulación parece que es

un mecanismo que se encuentra más extendido de lo que se pensaba ya que también se ha descrito en *P. aeruginosa* y *E. coli* (Heurlier *et al.*, 2004; Suzuki *et al.*, 2002; Ventre *et al.*, 2006).

Además de las propias autorregulaciones internas del sistema Gac, existen otros reguladores ajenos a esta cascada que pueden influir en ella. La inactivación del gen *kdgR* en el mutante *expA* (*gacA*) del fitopatógeno *Erwinia carotovora* subsp. *carotovora* provoca una reversión del fenotipo *expA*$^-$, restaurando parcialmente la producción de exo-enzimas y la virulencia (Hyytiäinen *et al.*, 2001). En el modelo propuesto por estos autores argumentan que ExpA y KdgR ejercen un efecto contrario en la expresión de *rsmA* y *rsmB*, de forma que KdgR aumenta la expresión de *rsmA* (proteína de unión a ARN) y disminuye la de *rsmB* (ARN regulador). Aarons y col. (2000) sugieren que un homólogo a KdgR podría también regular negativamente la expresión de *prrB* (homólogo a *rsmB*) en *P. fluorescens* F113 ya que presenta una secuencia de reconocimiento para KdgR en su zona promotora y otra en la zona codificante. Este modelo de regulación Gac parece que se complica en *P. aeruginosa* PAK porque se han encontrado dos sensores quinasa híbridos denominados LadS y RetS, importantes en el control central de la virulencia, que interactúan con la cascada reguladora Gac (Goodman *et al.*, 2004; Ventre *et al.*, 2006). Estos dos sensores actúan de forma inversa, mientras un mutante *retS* aumenta drásticamente la expresión del riborregulador *rsmZ* provocando un aumento en la formación de biopelículas (infección crónica) y una disminución en la expresión del TTSS (infección aguda), un mutante *ladS* presenta unos niveles de *rsmZ* similares a un mutante *gac* que conlleva una disminución en la formación de biopelículas y un aumento en el TTSS.

Con respecto a la regulación de los niveles de GacA y GacS no hay mucho descrito. En *P. fluorescens* Pf-5, se ha visto que GacA regula positivamente la acumulación de GacS (Whistler *et al.*, 1998). Esta regulación positiva de GacS por GacA puede ser un mecanismo para controlar la concentración relativa de las dos

proteínas, de forma que haya una estequiometría apropiada entre el sensor y el regulador. El factor sigma RpoS (σ^{s}) también regula la producción de GacS en esta bacteria, aunque al contrario que GacA, RpoS ejerce un efecto negativo sobre su expresión (Whistler *et al.*, 1998). RpoS parece que no es el único factor sigma que regula GacA/GacS, RpoN (σ^{54}) regula negativamente la expresión de *gacA* en *P. aeruginosa* a través de un represor desconocido (Heurlier *et al.*, 2003). A la luz de todos estos resultados parece que el sistema GacA/GacS está sujeto a una regulación bastante compleja.

REFERENCIAS

Aarons, S., Abbas, A., Adams, C., Fenton, A. & O'Gara, F. (2000). A regulatory RNA (PrrB RNA) modulates expression of secondary metabolite genes in *Pseudomonas fluorescens* F113. *J Bacteriol* **182**, 3913-3919.

Abraham, J. M., Freitag, C. S., Clements, J. R. & Eisenstein, B. I. (1985). An invertible element of DNA controls phase variation of type 1 fimbriae of *Escherichia coli*. *Proc Natl Acad Sci U S A* **82**, 5724-5727.

Achouak, W., Conrod, S., Cohen, V. & Heulin, T. (2004). Phenotypic variation of *Pseudomonas brassicacearum* as a plant root-colonization strategy. *Mol Plant-Microbe Interact* **17**, 872-879.

Alabouvette, C. (1986). *Fusarium* wilt suppressive soils from the chateaurenard region: Review of a 10 year study. *Agronomie* **6**, 273-284.

Alabouvette, C. & Couteaudier, Y. (1992). Biologial control of *Fusarium* wilts with non-pathogenic Fusaria. In *Biological Control of Plant Diseases*, pp. 415-426. Edited by E. C. Tjamos, R. J. Cook & G. C. Papavizas. New York: Plenum Press.

Allen, R. D. & Baumann, P. (1971). Structure and arrangement of flagella in species of genus *Beneckea* and *Photobacterium fischeria*. *J Bacteriol* **107**, 295-&.

Arora, S. K., Ritchings, B. W., Almira, E. C., Lory, S. & Ramphal, R. (1997). Transcriptional activator, FleQ, regulates mucin adhesion and flagellar gene expression in *Pseudomonas aeruginosa* in a cascade manner. *J Bacteriol* **179**, 5574-5581.

Arora, S. K., Ritchings, B. W., Almira, E. C., Lory, S. & Ramphal, R. (1998). The *Pseudomonas aeruginosa* flagellar cap protein, FliD, is responsible for mucin adhesion. *Infect Immun* **66**, 1000-1007.

Arora, S. K., Dasgupta, N., Lory, S. & Ramphal, R. (2000). Identification of two distinct types of flagellar cap proteins, FliD, in *Pseudomonas aeruginosa*. *Infect Immun* **68**, 1474-1479.

Arora, S. K., Bangera, M., Lory, S. & Ramphal, R. (2001). A genomic island in *Pseudomonas aeruginosa* carries the determinants of flagellin glycosylation. *Proc Natl Acad Sci U S A* **98**, 9342-9347.

Baker, C. S., Morozov, I., Suzuki, K., Romeo, T. & Babitzke, P. (2002). CsrA regulates glycogen biosynthesis by preventing translation of *glgC* in *Escherichia coli*. *Mol Microbiol* **44**, 1599-1610.

Bangera, M. G. & Thomashow, L. S. (1999). Identification and characterization of gene cluster for synthesis of the polyketide antibiotic 2,4-diacetylphloroglucinol from *Pseudomonas fluorescens* Q2-87. *J Bacteriol* **181**, 3155-3163.

Bardwell, J. C. A., Mcgovern, K. & Beckwith, J. (1991). Identification of a protein required for disulfide bond formation *in vivo*. *Cell* **67**, 581-589.

Barrett, C. F. & Parker, M. A. (2005). Prevalence of *Burkholderia* sp. nodule symbionts on four mimosoid legumes from Barro Colorado island Panama. *Syst Appl Microbiol* **28**, 57-65.

Bayliss, C. D., Field, D. & Moxon, E. R. (2001). The simple sequence contingency loci of *Haemophilus influenzae* and *Neisseria meningitidis*. *J Clin Invest* **107**, 657-662.

Bayliss, C. D., van de Ven, T. & Moxon, E. R. (2002). Mutations in *polI* but not *mutSLH* destabilize *Haemophilus influenzae* tetranucleotide repeats. *EMBO Journal* **21**, 1465-1476.

Bayliss, C. D., Dixon, K. M. & Moxon, E. R. (2004a). Simple sequence repeats (microsatellites): mutational mechanisms and contributions to bacterial pathogenesis. A meeting review. *FEMS Immunol Med Microbiol* **40**, 11-19.

Bayliss, C. D., Sweetman, W. A. & Moxon, E. R. (2004b). Mutations in *Haemophilus influenzae* mismatch repair genes increase mutation rates of dinucleotide repeat tracts but not dinucleotide repeat-driven pilin phase variation rates. *J Bacteriol* **186**, 2928-2935.

Bazin, M. J., Markham, P., Scott, E. M. & Lynch, J. M. (1990). Population dynamics and rhizosphere interactions. In *The Rhizosphere*, pp. 99-127. Chinchester: Wiley & Sons.

Belland, R. J. (1991). H-DNA formation by the coding repeat elements of neisserial *opa* genes. *Mol Microbiol* **5**, 2351-2360.

Blanton, K. J., Biswas, G. D., Tsai, J., Adams, J., Dyer, D. W., Davis, S. M., Koch, G. G., Sen, P. K. & Sparling, P. F. (1990). Genetic evidence that *Neisseria gonorrhoeae* produces specific receptors for transferrin and lactoferrin. *J Bacteriol* **172**, 5225-5235.

Bloemberg, G. V., Wijfjes, A. H. M., Lamers, G. E. M., Stuurman, N. & Lugtenberg, B. J. J. (2000). Simultaneous imaging of *Pseudomonas fluorescens* WCS365 populations expressing three different autofluorescent proteins in the rhizosphere: New perspectives for studying microbial communities. *Mol Plant-Microbe Interact* **13**, 1170-1176.

Blomfield, I. C., Calie, P. J., Eberhardt, K. J., Mcclain, M. S. & Eisenstein, B. I. (1993). Lrp stimulates phase variation of type 1 fimbriation in *Escherichia coli* K-12. *J Bacteriol* **175**, 27-36.

Blomfield, I. C., Kulasekara, D. H. & Eisenstein, B. I. (1997). Integration host factor stimulates both FimB- and FimE-mediated site-specific DNA inversion that controls phase variation of type 1 fimbriae expression in *Escherichia coli*. *Mol Microbiol* **23**, 705-717.

Blumer, C., Heeb, S., Pessi, G. & Haas, D. (1999). Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. *Proc Natl Acad Sci U S A* **96**, 14073-14078.

Boch, J., Joardar, V., Gao, L., Robertson, T. L., Lim, M. & Kunkel, B. N. (2002). Identification of *Pseudomonas syringae* pv. *tomato* genes induced during infection of *Arabidopsis thaliana*. *Mol Microbiol* **44**, 73-88.

Bolwerk, A., Lagopodi, A. L., Wijfjes, A. H. M., Lamers, G. E. M., Chin-A-Woeng, T. F. C., Lugtenberg, B. J. J. & Bloemberg, G. V. (2003). Interactions in the tomato rhizosphere of two *Pseudomonas* biocontrol strains with the phytopathogenic fungus *Fusarium oxysporum* f. sp *radicis-lycopersici*. *Mol Plant-Microbe Interact* **16**, 983-993.

Bolwerk, A., Lagopodi, A. L., Lugtenberg, B. J. J. & Bloemberg, G. V. (2005). Visualization of interactions between a pathogenic and a beneficial *Fusarium* strain during biocontrol of tomato foot and root rot. *Mol Plant-Microbe Interact* **18**, 710-721.

Bonfante, P. & Perotto, S. (1995). Strategies of arbuscular mycorrhizal fungi when infecting host plants. *New Phytol* **130**, 3-21.

Borst, P. (2003). Mechanisms of antigenic variation: an overview. In *Antigenic Variation*, pp. 1-15. Edited by A. Craig & A. Scherf. London (UK): Elsevier Academic Press.

Braun, V. (1997). Avoidance of iron toxicity through regulation of bacterial iron transport. *Biol Chem* **378**, 779-786.

Brazil, G. M., Kenefick, L., Callanan, M., Haro, A., de Lorenzo, V., Dowling, D. N. & O´Gara, F. (1995). Construction of a rhizosphere pseudomonad with potential to degrade polychlorinated biphenyls and detection of *bph* gene expression in the rhizosphere. *Appl Environ Microbiol* **61**, 1946-1952.

Brencic, A. & Winans, S. C. (2005). Detection of and response to signals involved in host-microbe interactions by plant-associated bacteria. *Microbiol Mol Biol Rev* **69**, 155-194.

Brodhagen, M., Henkels, M. D. & Loper, J. E. (2004). Positive autoregulation and signaling properties of pyoluteorin, an antibiotic produced by the biological control organism *Pseudomonas fluorescens* Pf-5. *Appl Environ Microbiol* **70**, 1758-1766.

Budzikiewicz, H. (1993). Secondary metabolites from fluorescent pseudomonads. *FEMS Microbiol Rev* **104**, 209-228.

Bull, C. T., Weller, D. M. & Thomashow, L. S. (1991). Relationship between root colonization and suppression of *Gaeumannomyces graminis* var. tritici by *Pseudomonas fluorescens* strain 2-79. *Phytopathology* **81**, 954-959.

Bull, C. T., Duffy, B., Voisard, C., Defago, G., Keel, C. & Haas, D. (2001). Characterization of spontaneous gacS and gacA regulatory mutants of *Pseudomonas fluorescens* biocontrol strain CHA0. *Antonie Van Leeuwenhoek* **79**, 327-336.

Burrowes, E., Baysse, C., Adams, C. & O'Gara, F. (2006). Influence of the regulatory protein RsmA on cellular functions in *Pseudomonas aeurginosa* PAO1, as revealed by transcriptome analysis. *Microbiol* **152**, 405-418.

Capdevila, S., Martínez-Granero, F. M., Sánchez-Contreras, M., Rivilla, R. & Martín, M. (2004). Analysis of *Pseudomonas fluorescens* F113 genes implicated in flagellar filament synthesis and their role in competitive root colonization. *Microbiol* **150**, 3889-3897.

Carvajal, M. M. C., Wijfjes, A. H. M., Mulders, I. H. M., Lugtenberg, B. J. J. & Bloemberg, G. V. (2002). Characterization of NADH dehydrogenases of *Pseudomonas fluorescens* WCS365 and their role in competitive root colonization. *Mol Plant-Microbe Interact* **15**, 662-671.

Casaz, P., Happel, A., Keithan, J., Read, D. L., Strain, S. R. & Levy, S. B. (2001). The *Pseudomonas fluorescens* transcription activator AdnA is required for adhesion and motility. *Microbiol* **147**, 355-361.

Cornelis, P. & Matthijs, S. (2002). Diversity of siderophore-mediated iron uptake systems in fluorescent pseudomonads: not only pyoverdines. *Environ Microbiol* **4**, 787-798.

Cornelissen, C. N. (2003). Transferrin-iron uptake by Gram-negative bacteria. *Front Biosci* **8**, D836-D847.

Cox, C. D., Rinehart, K. L., Moore, M. L. & Cook, J. C. (1981). Pyochelin: novel structure of an iron chelating growth promoter for *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **78**, 4256-4260.

Chabeaud, P., de Groot, A., Bitter, W., Tommassen, J., Heulin, T. & Achouak, W. (2001). Phase-variable expression of an operon encoding extracellular alkaline protease, a serine protease homolog, and lipase in *Pseudomonas brassicacearum*. *J Bacteriol* **183**, 2117-2120.

Chadsey, M. S., Karlinsey, J. E. & Hughes, K. T. (1998). The flagellar anti-sigma factor FlgM actively dissociates *Salmonella typhimurium* σ^{28} RNA polymerase holoenzyme. *Genes Dev* **12**, 3123-3136.

Chancey, S. T., Wood, D. W., Pierson, E. A. & Pierson, L. S. (2002). Survival of GacS/GacA mutants of the biological control bacterium *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere. *Appl Environ Microbiol* **68**, 3308-3314.

Chao, L., Vargas, C., Spear, B. B. & Cox, E. C. (1983). Transposable elements as mutator genes in evolution. *Nature* **303**, 633-635.

Chao, L. & McBroom, S. M. (1985). Evolution of transposable elements: an IS10 insertion increases fitness in *Escherichia coli*. *Mol Biol Evol* **2**, 359-369.

Chatterjee, A., Cui, Y. Y., Yang, H. L., Collmer, A., Alfano, J. R. & Chatterjee, A. K. (2003). GacA, the response regulator of a two-component system, acts as a master regulator in *Pseudomonas syringae* pv. *tomato* DC3000 by controlling regulatory RNA, transcriptional activators, and alternate sigma factors. *Mol Plant-Microbe Interact* **16**, 1106-1117.

Chen, W. M., Laevens, S., Lee, T. M., Coenye, T., De Vos, P., Mergeay, M. & Vandamme, P. (2001). *Ralstonia taiwanensis* sp nov., isolated from root nodules of *Mimosa* species and sputum of a cystic fibrosis patient. *Int J Syst Evol Microbiol* **51**, 1729-1735.

Chen, W. M., Moulin, L., Bontemps, C., Vandamme, P., Bena, G. & Boivin-Masson, C. (2003). Legume symbiotic nitrogen fixation by β-Proteobacteria is widespread in nature. *J Bacteriol* **185**, 7266-7272.

Chiang, P. & Burrows, L. L. (2003). Biofilm formation by hyperpiliated mutants of *Pseudomonas aeruginosa*. *J Bacteriol* **185**, 2374-2378.

Chilcott, G. S. & Hughes, K. T. (2000). Coupling of flagellar gene expression to flagellar assembly in *Salmonella enterica* serovar typhimurium and *Escherichia coli*. *Microbiol Mol Biol Rev* **64**, 694-708.

Chin-A-Woeng, T. F. C., de Priester, W., van der Bij, A. J. & Lugtenberg, B. J. J. (1997). Description of the colonization of a gnotobiotic tomato rhizosphere by *Pseudomonas fluorescens* biocontrol strain WCS365, using scanning electron microscopy. *Mol Plant-Microbe Interact* **10**, 79-86.

Chin-A-Woeng, T. F. C., Bloemberg, G. V., van der Bij, A. J. & other authors (1998). Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Mol Plant-Microbe Interact* **11**, 1069-1077.

Chin-A-Woeng, T. F. C., Bloemberg, G. V., Mulders, I. H. M., Dekkers, L. C. & Lugtenberg, B. J. J. (2000). Root colonization by phenazine-1-carboxamide-producing bacterium *Pseudomonas chlororaphis* PCL1391 is essential for biocontrol of tomato foot and root rot. *Mol Plant-Microbe Interact* **13**, 1340-1345.

Chin-A-Woeng, T. F. C., van den Broek, D., de Voer, G., van der Drift, K. M. G. M., Tuinman, S., Thomas-Oates, J. E., Lugtenberg, B. J. J. & Bloemberg, G. V. (2001). Phenazine-1-carboxamide production in the biocontrol strain *Pseudomonas chlororaphis* PCL1391 is regulated by multiple factors secreted into the growth medium. *Mol Plant-Microbe Interact* **14**, 969-979.

Chin-A-Woeng, T. F. C., Bloemberg, G. V. & Lugtenberg, B. J. J. (2003). Phenazines and their role in biocontrol by *Pseudomonas* bacteria. *New Phytol* **157**, 503-523.

Dailey, F. E. & Berg, H. C. (1993). Mutants in disulfide bond formation that disrupt flagellar assembly in *Escherichia coli*. *Proc Natl Acad Sci U S A* **90**, 1043-1047.

Darzins, A. & Russell, M. A. (1997). Molecular genetic analysis of type-4 pilus biogenesis and twitching motility using *Pseudomonas aeruginosa* as a model system - a review. *Gene* **192**, 109-115.

Dasgupta, N. & Ramphal, R. (2001). Interaction of the antiactivator FleN with the transcriptional activator FleQ regulates flagellar number in *Pseudomonas aeruginosa*. *J Bacteriol* **183**, 6636-6644.

Dasgupta, N., Ferrell, E. P., Kanack, K. J., West, S. E. H. & Ramphal, R. (2002). *fleQ*, the gene encoding the major flagellar regulator of *Pseudomonas aeruginosa*, is σ^{70} dependent and is downregulated by Vfr, a homolog of *Escherichia coli* cyclic AMP receptor protein. *J Bacteriol* **184**, 5240-5250.

Dasgupta, N., Wolfgang, M. C., Goodman, A. L., Arora, S. K., Jyot, J., Lory, S. & Ramphal, R. (2003). A four-tiered transcriptional regulatory circuit controls flagellar biogenesis in *Pseudomonas aeruginosa*. *Mol Microbiol* **50**, 809-824.

Dasgupta, N., Arora, S. K. & Ramphal, R. (2004). The flagellar system of *Pseudomonas aeruginosa*. In *Pseudomonas*, pp. 675-698. Edited by J. L. Ramos. New York: Kluwer Academic/Plenum Publishers.

de Weert, S., Vermeiren, H., Mulders, I. H. M., Kuiper, I., Hendrickx, N., Bloemberg, G. V., Vanderleyden, J., de Mot, R. & Lugtenberg, B. J. J. (2002). Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by *Pseudomonas fluorescens*. *Mol Plant-Microbe Interact* **15**, 1173-1180.

de Weger, L. A., van der vlugt, C. I. M., Wijfjes, A. H. M., Bakker, P. A. H. M., Schippers, B. & Lugtenberg, B. (1987). Flagella of a plant-growth-stimulating *Pseudomonas fluorescens* strain are required for colonization of potato roots. *J Bacteriol* **169**, 2769-2773.

de Weger, L. A., Vanderbij, A. J., Dekkers, L. C., Simons, M., Wijffelman, C. A. & Lugtenberg, B. J. J. (1995). Colonization of the rhizosphere of crop plants by plant-beneficial pseudomonads. *FEMS Microbiol Ecol* **17**, 221-227.

Dekkers, L. C., Bloemendaal, C. J. P., de Weger, L. A., Wijffelman, C. A., Spaink, H. P. & Lugtenberg, B. J. J. (1998a). A two-component system plays an important role in the root-colonizing ability of *Pseudomonas fluorescens* strain WCS365. *Mol Plant-Microbe Interact* **11**, 45-56.

Dekkers, L. C., Phoelich, C. C., van der Fits, L. & Lugtenberg, B. J. J. (1998b). A site-specific recombinase is required for competitive root colonization by *Pseudomonas fluorescens* WCS365. *Proc Natl Acad Sci U S A* **95**, 7051-7056.

Dekkers, L. C., van der Bij, A. J., Mulders, I. H. M., Phoelich, C. C., Wentwoord, R. A. R., Glandorf, D. C. M., Wijffelman, C. A. & Lugtenberg, B. J. J. (1998c). Role of the O-antigen of lipopolysaccharide, and possible roles of growth rate and of NADH : ubiquinone oxidoreductase (Nuo) in competitive tomato root-tip colonization by *Pseudomonas fluorescens* WCS365. *Mol Plant-Microbe Interact* **11**, 763-771.

Dekkers, L. C., Mulders, I. H. M., Phoelich, C. C., Chin-A-Woeng, T. F. C., Wijfjes, A. H. M. & Lugtenberg, B. J. J. (2000). The *sss* colonization gene of the tomato-*Fusarium oxysporum F.* sp *radicis-lycopersici* biocontrol strain *Pseudomonas fluorescens* WCS365

can improve root colonization of other wild-type *Pseudomonas* spp. bacteria. *Mol Plant-Microbe Interact* **13**, 1177-1183.

Delany, I., Sheehan, M. M., Fenton, A., Bardin, S., Aarons, S. & O'Gara, F. (2000). Regulation of production of the antifungal metabolite 2,4-diacetylphloroglucinol in *Pseudomonas fluorescens* F113: genetic analysis of *phlF* as a transcriptional repressor. *Microbiol* **146**, 537-546.

Denamur, E. & Matic, I. (2006). Evolution of mutation rates in bacteria. *Mol Microbiol* **60**, 820-827.

Deretic, V., Schurr, M. J., Boucher, J. C. & Martin, D. W. (1994). Conversion of *Pseudomonas aeruginosa* to mucoidy in cystic fibrosis: environmental stress and regulation of bacterial virulence by alternative sigma factors. *J Bacteriol* **176**, 2773-2780.

Déziel, E., Comeau, Y. & Villemur, R. (2001). Initiation of biofilm formation by *Pseudomonas aeruginosa* 57RP correlates with emergence of hyperpiliated and highly adherent phenotypic variants deficient in swimming, swarming, and twitching motilities. *J Bacteriol* **183**, 1195-1204.

Dörr, J., Hurek, T. & Reinhold-Hurek, B. (1998). Type IV pili are involved in plant-microbe and fungus-microbe interactions. *Mol Microbiol* **30**, 7-17.

Drenkard, E. & Ausubel, F. M. (2002). *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature* **416**, 740-743.

Dubrac, S. & Touati, D. (2000). Fur positive regulation of iron superoxide dismutase in *Escherichia coli*: functional analysis of the *sodB* promoter. *J Bacteriol* **182**, 3802-3808.

Dubuis, C., Rolli, J., Lutz, M., Défago, G. & Haas, D. (2006). Thiamine-auxotrophic mutants of *Pseudomonas fluorescens* CHA0 are defective in cell-cell signaling and biocontrol factor expression. *Appl Environ Microbiol* **72**, 2606-2613.

Duffy, B. K. & Défago, G. (1999). Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. *Appl Environ Microbiol* **65**, 2429-2438.

Duffy, B. K. & Défago, G. (2000). Controlling instability in *gacS-gacA* regulatory genes during inoculant production of *Pseudomonas fluorescens* biocontrol strains. *Appl Environ Microbiol* **66**, 3142-3150.

Duijff, B. J., Pouhair, D., Olivain, C., Alabouvette, C. & Lemanceau, P. (1998). Implication of systemic induced resistance in the suppression of *Fusarium* wilt of tomato by *Pseudomonas fluorescens* WCS417r and by nonpathogenic *Fusarium oxysporum* Fo47. *Eur J Plant Pathol* **104**, 903-910.

Dybvig, K. (1993). DNA rearrangements and phenotypic switching in prokaryotes. *Mol Microbiol* **10**, 465-471.

Ellis, R. J., Timms-Wilson, T. M., Beringer, J. E., Rhodes, D., Renwick, A., Stevenson, L. & Bailey, M. J. (1999). Ecological basis for biocontrol of damping-off disease by *Pseudomonas fluorescens* 54/96. *J Appl Microbiol* **87**, 454-463.

Espinosa-Urgel, M., Salido, A. & Ramos, J. L. (2000). Genetic analysis of functions involved in adhesion of *Pseudomonas putida* to seeds. *J Bacteriol* **182**, 2363-2369.

Fedi, S., Tola, E., Moenne Loccoz, Y., Dowling, D. N., Smith, L. M. & O´Gara, F. (1997). Evidence for signaling between the phytopathogenic fungus *Pythium ultimum* and *Pseudomonas fluorescens* F113: *P. ultimum* represses the expression of genes in *P. fluorescens* F113, resulting in altered ecological fitness. *Appl Environ Microbiol* **63**, 4261-4266.

Feldman, M., Bryan, R., Rajan, S., Scheffler, L., Brunnert, S., Tang, H. & Prince, A. (1998). Role of flagella in pathogenesis of *Pseudomonas aeruginosa* pulmonary infection. *Infect Immun* **66**, 43-51.

Fenton, A. M., Stephens, P. M., Crowley, J., O´Callaghan, M. & O´Gara, F. (1992). Exploitation of gene(s) involved in 2,4-diacetylphloroglucinol biosynthesis to confer a new biocontrol capability to a *Pseudomonas* strain. *Appl Environ Microbiol* **58**, 3873-3878.

Fortune, D. R., Suyemoto, M. & Altier, C. (2006). Identification of *csrC* and characterization of its role in epithelial cell invasion in *Salmonella enterica* serovar *typhimurium*. *Infect Immun* **74**, 331-339.

Fraser, G. M., Claret, L., Furness, R., Gupta, S. & Hughes, C. (2002). Swarming-coupled expression of the *Proteus mirabilis hpmBA* haemolysin operon. *Microbiol* **148**, 2191-2201.

Frey-Klett, P., Pierrat, J. C. & Garbaye, J. (1997). Location and survival of mycorrhiza helper *Pseudomonas fluorescens* during establishment of ectomycorrhizal symbiosis between *Laccaria bicolor* and Douglas fir. *Appl Environ Microbiol* **63**, 139-144.

Frisk, A., Jyot, J., Arora, S. K. & Ramphal, R. (2002). Identification and functional characterization of *flgM*, a gene encoding the anti-sigma 28 factor in *Pseudomonas aeruginosa*. *J Bacteriol* **184**, 1514-1521.

Fuchs, J. G., Moenne-Loccoz, Y. & Defago, G. (1997). Nonpathogenic *Fusarium oxysporum* strain Fo47 induces resistance to *Fusarium* wilt in tomato. *Plant Dis* **81**, 492-496.

Gaffney, T. D., Lam, S. T., Ligon, J. & other authors (1994). Global regulation of expression of antifungal factors by a *Pseudomonas fluorescens* biological control strain. *Mol Plant-Microbe Interact* **7**, 455-463.

Garbaye, J. (1994). Helper bacteria: a new dimension to the mycorrhizal symbiosis. *New Phytol* **128**, 197-210.

Geis, G., Suerbaum, S., Forsthoff, B., Leying, H. & Opferkuch, W. (1993). Ultrastructure and biochemical studies of the flagellar sheath of *Helicobacter pylori*. *J Med Microbiol* **38**, 371-377.

Gerhardson, B. (2002). Biological substitutes for pesticides. *Trends Biotechnol* **20**, 338-343.

Gilardi, G. (1985). Pseudomonas. In *Manual of Clinical Microbiology*, pp. 350-372. Edited by E. Lennette, A. Balows, W. Hausler & H. Shadomy. Washington, DC (USA): ASM Press.

Givaudan, A. & Lanois, A. (2000). *flhDC*, the flagellar master operon of *Xenorhabdus nematophilus*: requirement for motility, lipolysis, extracellular hemolysis, and full virulence in insects. *J Bacteriol* **182**, 107-115.

Gober, J. W. & England, J. C. (2000). Regulation of flagellum biogenesis and motility in *Caulobacter*. In *Prokaryotic Development*, pp. 319-340. Edited by Y. V. Brun & L. J. Schimkets. Whashington, D.C. (USA): ASM Press.

González Barrios, A. F., Zuo, R. J., Hashimoto, Y., Yang, L., Bentley, W. E. & Wood, T. K. (2006). Autoinducer 2 controls biofilm formation in *Escherichia coli* through a novel motility quorum-sensing regulator (MqsR, B3022). *J Bacteriol* **188**, 305-316.

Goodier, R. I. & Ahmer, B. M. M. (2001). SirA orthologs affect both motility and virulence. *J Bacteriol* **183**, 2249-2258.

Goodman, A. L., Kulasekara, B., Rietsch, A., Boyd, D., Smith, R. S. & Lory, S. (2004). A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*. *Developmental Cell* **7**, 745-754.

Goodman, M. F. (2002). Error-prone repair DNA polymerases in prokaryotes and eukaryotes. *Annu Rev Biochem* **71**, 17-50.

Graupner, S., Frey, V., Hashemi, R., Lorenz, M. G., Brandes, G. & Wackernagel, W. (2000). Type IV pilus genes *pilA* and *pilC* of *Pseudomonas stutzeri* are required for natural genetic transformation, and *pilA* can be replaced by corresponding genes from nontransformable species. *J Bacteriol* **182**, 2184-2190.

Gutterson, N. (1990). Microbial fungicides: recent approaches to elucidating mechanisms. *Crit Rev Biotechnol* **10**, 69-91.

Haas, D., Keel, C. & Reimmann, C. (2002). Signal transduction in plant-beneficial rhizobacteria with biocontrol properties. *Antonie Van Leeuwenhoek* **81**, 385-395.

Haas, D. & Keel, C. (2003). Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. *Annu Rev Phytopathol* **41**, 117-153.

Haas, D. & Défago, G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nature Reviews Microbiology* **3**, 307-319.

Hahn, H. P. (1997). The type-4 pilus is the major virulence-associated adhesin of *Pseudomonas aeruginosa* - a review. *Gene* **192**, 99-108.

Hall, P. G. & Krieg, N. R. (1984). Application of the indirect immunoperoxidase stain technique to the flagella of *Azospirillum brasilense*. *Appl Environ Microbiol* **47**, 433-435.

Han, B., Pain, A. & Johnstone, K. (1997). Spontaneous duplication of a 661 bp element within a two-component sensor regulator gene causes phenotypic switching in colonies of *Pseudomonas tolaasii*, cause of brown blotch disease of mushrooms. *Mol Microbiol* **25**, 211-218.

Handfield, M., Lehoux, D. E., Sanschagrin, F., Mahan, M. J., Woods, D. E. & Levesque, R. C. (2000). *In vivo*-induced genes in *Pseudomonas aeruginosa*. *Infect Immun* **68**, 2359-2362.

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I. & Lorito, M. (2004). *Trichoderma* species: Opportunistic, avirulent plant symbionts. *Nature Reviews Microbiology* **2**, 43-56.

Harshey, R. M. (1994). Bees aren´t the only ones: swarming in gram-negative bacteria. *Mol Microbiol* **13**, 389-394.

Hartmann, A., Gantner, S., Schuhegger, R., Steidle, A., Durr, C., Schmid, M., Dazzo, F., Eberl, L. & Langebartels, C. (2004). *N*-acyl homoserine lactones of rhizosphere bacteria trigger systemic resistance in tomato plants. In *Proceedings of the 11th International Congress on Molecular Plant-Microbe Interactions*, pp. 554-556. Edited by I. Tikhonovich, B. Lugtenberg & N. Provorov. St. Paul, Minnesota (USA): International Society for Molecular Plant-Microbe Interaction.

Harwood, C. S., Fosnaugh, K. & Dispensa, M. (1989). Flagellation of *Pseudomonas putida* and analysis of its motile behavior. *J Bacteriol* **171**, 4063-4066.

Häuβler, S., Ziegler, I., Lottel, A., von Gotz, F., Rohde, M., Wehmhohner, D., Saravanamuthu, S., Tummler, B. & Steinmetz, I. (2003). Highly adherent small-colony variants of *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *J Med Microbiol* **52**, 295-301.

Heeb, S. & Haas, D. (2001). Regulatory roles of the GacS/GacA two-component system in plant-associated and other Gram-negative bacteria. *Mol Plant-Microbe Interact* **14**, 1351-1363.

Heeb, S., Blumer, C. & Haas, D. (2002). Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0. *J Bacteriol* **184**, 1046-1056.

Henderson, I. R., Owen, P. & Nataro, J. P. (1999). Molecular switches - the ON and OFF of bacterial phase variation. *Mol Microbiol* **33**, 919-932.

Heurlier, K., Dénervaud, V., Pessi, G., Reimmann, C. & Haas, D. (2003). Negative control of quorum sensing by RpoN (σ^{54}) in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **185**, 2227-2235.

Heurlier, K., Williams, F., Heeb, S., Dormond, C., Pessi, G., Singer, D., Camara, M., Williams, P. & Haas, D. (2004). Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/*rsmZ* system in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **186**, 2936-2945.

Higgs, P. I., Myers, P. S. & Postle, K. (1998). Interactions in the TonB-dependent energy transduction complex: ExbB and ExbD form homomultimers. *J Bacteriol* **180**, 6031-6038.

Horino, A., Sasaki, Y., Sasaki, T. & Kenri, T. (2003). Multiple promoter inversions generate surface antigenic variation in *Mycoplasma penetrans*. *J Bacteriol* **185**, 231-242.

Howie, W. J., Cook, R. J. & Weller, D. M. (1987). Effects of soil matric potential and cell motility on wheat root colonization by fluorescent pseudomonads suppressive to take-all. *Phytopathology* **77**, 286-292.

Hrabak, E. M. & Willis, D. K. (1992). The *lemA* gene required for pathogenicity of *Pseudomonas syringae* pv. *syringae* on bean is a member of a family of two-component regulators. *J Bacteriol* **174**, 3011-3020.

Hughes, K. T., Gillen, K. L., Semon, M. J. & Karlinsey, J. E. (1993). Sensing structural intermediates in bacterial flagellar assembly by export of a negative regulator. *Science* **262**, 1277-1280.

Hyytiäinen, H., Montesano, M. & Palva, E. T. (2001). Global regulators ExpA (GacA) and KdgR modulate extracellular enzyme gene expression through the RsmA-*rsmB* system in *Erwinia carotovora* subsp *carotovora*. *Mol Plant-Microbe Interact* **14**, 931-938.

Iavicoli, A., Boutet, E., Buchala, A. & Metraux, J. P. (2003). Induced systemic resistance in *Arabidopsis thaliana* in response to root inoculation with *Pseudomonas fluorescens* CHA0. *Mol Plant-Microbe Interact* **16**, 851-858.

Ilves, H., Horak, R. & Kivisaar, M. (2001). Involvement of σ^{s} in starvation-induced transposition of *Pseudomonas putida* transposon Tn4652. *J Bacteriol* **183**, 5445-5448.

Jander, G., Rahme, L. G. & Ausubel, F. M. (2000). Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. *J Bacteriol* **182**, 3843-3845.

Kamilova, F., Validov, S., Azarova, T., Mulders, I. & Lugtenberg, B. (2005). Enrichment for enhanced competitive plant root tip colonizers selects for a new class of biocontrol bacteria. *Environ Microbiol* **7**, 1809-1817.

Kang, B. R., Yang, K. Y., Cho, B. H., Han, T. H., Kim, I. S., Lee, M. C., Anderson, A. J. & Kim, Y. C. (2006). Production of indole-3-acetic acid in the plant-beneficial strain

Pseudomonas chlororaphis O6 is negatively regulated by the global sensor kinase GacS. *Curr Microbiol* **52**, 473-476.

Karlinsey, J. E., Tanaka, S., Bettenworth, V., Yamaguchi, S., Boos, W., Aizawa, S. I. & Hughes, K. T. (2000). Completion of the hook-basal body complex of the *Salmonella typhimurium* flagellum is coupled to FlgM secretion and *fliC* transcription. *Mol Microbiol* **37**, 1220-1231.

Karlson, U., Dowling, D., O´Gara, F., Rivilla, R., Bittens, M., Francesconi, S., Pritchard, H. & Pederson, H. C. (1998). Development of self-contained plant/GMM system for soil bioremediation. In *Past, Present and Future Risk Assessment when using GMOs*, pp. 23-31. Edited by G. E. d. Vries. Overschild, NL: Probio Partners.

Kay, E., Dubuis, C. & Haas, D. (2005). Three small RNAs jointly ensure secondary metabolism and biocontrol in *Pseudomonas fluorescens* CHA0. *Proc Natl Acad Sci U S A* **102**, 17136-17141.

Kay, E., Humair, B., Dénervaud, V., Riedel, K., Spahr, S., Eberl, L., Valverde, C. & Haas, D. (2006). Two GacA-dependent small RNAs modulate the quorum-sensing response in *Pseudomonas aeruginosa*. *J Bacteriol* **188**, 6026-6033.

Keel, C., Voisard, C., Berling, C. H., Kahr, G. & Défago, G. (1989). Iron sufficiency, a prerequisite for the suppression of tobacco black root rot by *Pseudomonas fluorescens* strain CHA0 under gnotobiotic conditions. *Phytopathology* **79**, 584-589.

Keel, C., Schnider, U., Maurhofer, M., Voisard, C., Laville, J., Burger, U., Wirthner, P., Haas, D. & Défago, G. (1992). Suppression of root diseases by *Pseudomonas fluorescens* CHA0: importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. *Mol Plant-Microbe Interact* **5**, 4-13.

Khan, S. R., Mavrodi, D. V., Jog, G. J., Suga, H., Thomashow, L. S. & Farrand, S. K. (2005). Activation of the *phz* operon of *Pseudomonas fluorescens* 2-79 requires the LuxR homolog PhzR, *N*-(3-OH-hexanoyl)-L-homoserine lactone produced by the LuxI homolog PhzI, and a *cis*-acting *phz* box. *J Bacteriol* **187**, 6517-6527.

Kim, D. J., Boylan, B., George, N. & Forst, S. (2003). Inactivation of *ompR* promotes precocious swarming and *flhDC* expression in *Xenorhabdus nematophila*. *J Bacteriol* **185**, 5290-5294.

Kim, Y. K. & McCarter, L. L. (2000). Analysis of the polar flagellar gene system of *Vibrio parahaemolyticus*. *J Bacteriol* **182**, 3693-3704.

Kinscherf, T. G. & Willis, D. K. (1999). Swarming by *Pseudomonas syringae* B728a requires *gacS* (*lemA*) and *gacA* but not the acyl-homoserine lactone biosynthetic gene *ahlI*. *J Bacteriol* **181**, 4133-4136.

Kistner, C. & Parniske, M. (2002). Evolution of signal transduction in intracellular symbiosis. *Trends Plant Sci* **7**, 511-518.

Kivisaar, M. (2003). Stationary phase mutagenesis: mechanisms that accelerate adaptation of microbial populations under environmental stress. *Environ Microbiol* **5**, 814-827.

Kloek, A. P., Brooks, D. M. & Kunkel, B. N. (2000). A *dsbA* mutant *Pseudomonas syringae* exhibits reduced virulence and partial impairment of type III secretion. *Mol Plant Pathol* **1**, 139-150.

Kloepper, J. W. & Schroth, M. (1978). Plant growth-promoting rhizobacteria on radishes. In *Proceedings of the 4th International Conference on Plant Pathogenic Bacteria*, pp. 879-882. Tours (France): Gilbert-Clarey.

Kloepper, J. W., Leong, J., Teintze, M. & Schroth, M. N. (1980). Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. *Nature* **286**, 885-886.

Kloepper, J. W., Lifshitz, R. & Zablotowicz, R. M. (1989). Free-living bacterial inocula for enhancing crop productivity. *Trends Biotechnol* **7**, 39-44.

Koch, B., Nielsen, T. H., Sorensen, D., Andersen, J. B., Christophersen, C., Molin, S., Givskov, M., Sorensen, J. & Nybroe, O. (2002). Lipopeptide production in *Pseudomonas* sp. strain DSS73 is regulated by components of sugar beet seed exudate via the Gac two-component regulatory system. *Appl Environ Microbiol* **68**, 4509-4516.

Koebnik, R. (2005). TonB-dependent trans-envelope signalling: the exception or the rule? *Trends Microbiol* **13**, 343-347.

Köhler, T., Curty, L. K., Barja, F., van Delden, C. & Pechere, J. C. (2000). Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. *J Bacteriol* **182**, 5990-5996.

Koster, M., Ovaa, W., Bitter, W. & Weisbeek, P. (1995). Multiple outer membrane receptors for uptake of ferric pseudobactins in *Pseudomonas putida* WCS358. *Mol Gen Genet* **248**, 735-743.

Köster, W. (2001). ABC transporter-mediated uptake of iron, siderophores, heme and vitamin B_{12}. *Res Microbiol* **152**, 291-301.

Kraus, J. & Loper, J. E. (1992). Lack of evidence for a role of antifungal metabolite production by *Pseudomonas fluorescens* Pf-5 in biological control of *Pythium* damping-off of cucumber. *Phytopathology* **82**, 264-271.

Kresse, A. U., Dinesh, S. D., Larbig, K. & Römling, U. (2003). Impact of large chromosomal inversions on the adaptation and evolution of *Pseudomonas aeruginosa* chronically colonizing cystic fibrosis lungs. *Mol Microbiol* **47**, 145-158.

Kuiper, I., Bloemberg, G. V., Noreen, S., Thomas-Oates, J. E. & Lugtenberg, B. J. J. (2001). Increased uptake of putrescine in the rhizosphere inhibits competitive root colonization by *Pseudomonas fluorescens* strain WCS365. *Mol Plant-Microbe Interact* **14**, 1096-1104.

Kuiper, I., Kravchenko, L. V., Bloemberg, G. V. & Lugtenberg, B. J. J. (2002). *Pseudomonas putida* strain PCL1444, selected for efficient root colonization and naphtalene degradation, effectively utilizes root exudate components. *Mol Plant-Microbe Interact* **15**, 734-741.

Kutsukake, K. (1997). Autogenous and global control of the flagellar master operon, *flhD*, in *Salmonella typhimurium*. *Mol Gen Genet* **254**, 440-448.

Laville, J., Voisard, C., Keel, C., Maurhofer, M., Defago, G. & Haas, D. (1992). Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root-rot of tobacco. *Proc Natl Acad Sci U S A* **89**, 1562-1566.

Laville, J., Blumer, C., Von Schroetter, C., Gaia, V., Défago, G., Keel, C. & Haas, D. (1998). Characterization of the *hcnABC* gene cluster encoding hydrogen cyanide synthase and anaerobic regulation by ANR in the strictly aerobic biocontrol agent *Pseudomonas fluorescens* CHA0. *J Bacteriol* **180**, 3187-3196.

Ledgham, F., Ventre, I., Soscia, C., Foglino, M., Sturgis, J. N. & Lazdunski, A. (2003). Interactions of the quorum sensing regulator QscR: interaction with itself and the other regulators of *Pseudomonas aeruginosa* LasR and RhlR. *Mol Microbiol* **48**, 199-210.

Lee, S. W. & Cooksey, D. A. (2000). Genes expressed in *Pseudomonas putida* during colonization of a plant-pathogenic fungus. *Appl Environ Microbiol* **66**, 2764-2772.

Leeman, M., Denouden, E. M., van Pelt, J. A., Dirkx, F. P. M., Steijl, H., Bakker, P. A. H. M. & Schippers, B. (1996). Iron availability affects induction of systemic resistance to *Fusarium* wilt of radish by *Pseudomonas fluorescens*. *Phytopathology* **86**, 149-155.

Lemanceau, P. & Alabouvette, C. (1990). Biological control of *Fusarium* diseases by the association of fluorescent *Pseudomonas* and non-pathogenic *Fusarium*. In *Plant Growth Promoting Rhizobacteria*, pp. 45-50. Edited by C. Keel, B. Knoller & G. Défago. Interlaken (Switzerland): International Union of Biological Sciences.

Lenz, D. H., Miller, M. B., Zhu, J., Kulkarni, R. V. & Bassler, B. L. (2005). CsrA and three redundant small RNAs regulate quorum sensing in *Vibrio cholerae*. *Mol Microbiol* **58**, 1186-1202.

Leoni, L., Orsi, N., de Lorenzo, V. & Visca, P. (2000). Functional analysis of PvdS, an iron starvation sigma factor of *Pseudomonas aeruginosa*. *J Bacteriol* **182**, 1481-1491.

Levinson, G. & Gutman, G. A. (1987). Slipped-strand mispairing: a major mechanism for DNA sequence evolution. *Mol Biol Evol* **4**, 203-221.

Liao, C. H., McCallus, D. E., Wells, J. M., Tzean, S. S. & Kang, G. Y. (1996). The *repB* gene required for production of extracellular enzymes and fluorescent siderophores in *Pseudomonas viridiflava* is an analog of the *gacA* gene of *Pseudomonas syringae*. *Can J Microbiol* **42**, 177-182.

Liao, C. H., McCallus, D. E., Fett, W. F. & Kang, Y. G. (1997). Identification of gene loci controlling pectate lyase production and soft-rot pathogenicity in *Pseudomonas marginalis*. *Can J Microbiol* **43**, 425-431.

Liu, M. Y., Yang, H. H. & Romeo, T. (1995). The product of the pleiotropic *Escherichia coli* gene *csrA* modulates glycogen biosynthesis via effects on mRNA stability. *J Bacteriol* **177**, 2663-2672.

Liu, X. Y. & Matsumura, P. (1994). The FlhD/FlhC complex, a transcriptional activator of the *Escherichia coli* flagellar class II operons. *J Bacteriol* **176**, 7345-7351.

Liu, Y., Cui, Y. Y., Mukherjee, A. & Chatterjee, A. K. (1998). Characterization of a novel RNA regulator of *Erwinia carotovora* ssp. *carotovora* that controls production of extracellular enzymes and secondary metabolites. *Mol Microbiol* **29**, 219-234.

Loper, J. E., Suslow, T. V. & Schroth, M. N. (1984). Lognormal distribution of bacterial populations in the rhizosphere. *Phytopathology* **74**, 1454-1460.

Loper, J. E. & Buyer, J. S. (1991). Siderophores in microbial interactions on plant surfaces. *Mol Plant-Microbe Interact* **4**, 5-13.

Loper, J. E. & Henkels, M. D. (1997). Availability of iron to *Pseudomonas fluorescens* in rhizosphere and bulk soil evaluated with an ice nucleation reporter gene. *Appl Environ Microbiol* **63**, 99-105.

Loper, J. E. & Henkels, M. D. (1999). Utilization of heterologous siderophores enhances levels of iron available to *Pseudomonas putida* in the rhizosphere. *Appl Environ Microbiol* **65**, 5357-5363.

Lovett, S. T. & Feschenko, V. V. (1996). Stabilization of diverged tandem repeats by mismatch repair: Evidence for deletion formation via a misaligned replication intermediate. *Proc Natl Acad Sci U S A* **93**, 7120-7124.

Lugtenberg, B. J. J., Kravchenko, L. V. & Simons, M. (1999). Tomato seed and root exudate sugars: composition, utilization by *Pseudomonas* biocontrol strains and role in rhizosphere colonization. *Environ Microbiol* **1**, 439-446.

Lugtenberg, B. J. J., Dekkers, L. & Bloemberg, G. V. (2001). Molecular determinants of rhizosphere colonization by *Pseudomonas*. *Annu Rev Phytopathol* **39**, 461-490.

Lugtenberg, B. J. J. & Bloemberg, G. V. (2004). Life in the rhizosphere. In *Pseudomonas*. Edited by J. L. Ramos. New York: Kluwer Academic/Plenum Publishers.

Macnab, R. B. (1996). Flagella and motility. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, pp. 123-145. Edited by F. Neidhardt, R. Curtiss(III), J. Ingraham, E. Lin, K. Low, B. Magasanik & other authors. Washington, DC (USA): ASM Press.

Macnab, R. M. (2003). How bacteria assemble flagella. *Annu Rev Microbiol* **57**, 77-100.

Mahan, M. J., Slauch, J. M. & Mekalanos, J. J. (1993). Selection of bacterial virulence genes that are specifically induced in host tissues. *Science* **259**, 686-688.

Mahenthiralingam, E., Campbell, M. E. & Speert, D. P. (1994). Nonmotility and phagocytic resistance of *Pseudomonas aeruginosa* isolates from chronically colonized patients with cystic fibrosis. *Infect Immun* **62**, 596-605.

Marco, M. L., Legac, J. & Lindow, S. E. (2003). Conditional survival as a selection strategy to identify plant-inducible genes of *Pseudomonas syringae*. *Appl Environ Microbiol* **69**, 5793-5801.

Marco, M. L., Legac, J. & Lindow, S. E. (2005). *Pseudomonas syringae* genes induced during colonization of leaf surfaces. *Environ Microbiol* **7**, 1379-1391.

Marinus, M. G. (1996). Methylation of DNA. In *Escherichia coli and Salmonelk: Cellular and Molecular Biology*, pp. 782-791. Edited by F. C. Neidhardt, R. Curtiss, J. L. I. Ingraham, E. C. C. Lin, K. B. Low, B. B. Magasanik & other authors. Washington, DC (USA): ASM Press.

Martínez-Granero, F., Capdevila, S., Sánchez-Contreras, M., Martín, M. & Rivilla, R. (2005). Two site-specific recombinases are implicated in phenotypic variation and competitive rhizosphere colonization in *Pseudomonas fluorescens*. *Microbiol* **151**, 975-983.

Martínez-Granero, F., Rivilla, R. & Martin, M. (2006). Rhizosphere selection of highly motile phenotypic variants of *Pseudomonas fluorescens* with enhanced competitive colonization ability. *Appl Environ Microbiol* **72**, 3429-3434.

Massé, E. & Gottesman, S. (2002). A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. *Proc Natl Acad Sci U S A* **99**, 4620-4625.

Massé, E. & Arguin, W. (2005). Ironing out the problem: new mechanisms of iron homeostasis. *Trends Biochem Sci* **30**, 462-468.

Maurhofer, M., Hase, C., Meuwly, P., Métraux, J. P. & Défago, G. (1994a). Induction of systemic resistance of tobacco to tobacco necrosis virus by the root-colonizing *Pseudomonas fluorescens* strain CHA0: influence of the *gacA* gene and of pyoverdine production. *Phytopathology* **84**, 139-146.

Maurhofer, M., Keel, C., Haas, D. & Défago, G. (1994b). Pyoluteorin production by *Pseudomonas fluorescens* strain CHA0 is involved in the suppression of *Pythium* damping-off of cress but not of cucumber. *Eur J Plant Pathol* **100**, 221-232.

Mavrodi, O. V., Mavrodi, D. V., Park, A. A., Weller, D. M. & Thomashow, L. S. (2006a). The role of *dsbA* in colonization of the wheat rhizosphere by *Pseudomonas fluorescens* Q8r1-96. *Microbiol* **152**, 863-872.

Mavrodi, O. V., Mavrodi, D. V., Weller, D. M. & Thomashow, L. S. (2006b). The role of *ptsP*, *orfT*, and *sss* recombinase in root colonization by *Pseudomonas fluorescens* Q8r1-96. *Appl Environ Microbiol*, doi:10.1128/AEM.01215-01206.

McCarter, L. L. (2004). Dual flagellar systems enable motility under different circumstances. *J Mol Microbiol Biotechnol* **7**, 18-29.

McClain, J., Rollo, D. R., Rushing, B. G. & Bauer, C. E. (2002). *Rhodospirillum centenum* utilizes separate motor and switch components to control lateral and polar flagellum rotation. *J Bacteriol* **184**, 2429-2438.

McClain, M. S., Blomfield, I. C. & Eisenstein, B. I. (1991). Roles of *fimB* and *fimE* in site-specific DNA inversion associated with phase variation of type 1 fimbriae in *Escherichia coli*. *J Bacteriol* **173**, 5308-5314.

McClain, M. S., Blomfield, I. C., Eberhardt, K. J. & Eisenstein, B. I. (1993). Inversion-independent phase variation of type 1 fimbriae in *Escherichia coli*. *J Bacteriol* **175**, 4335-4344.

Mehr, I. J. & Seifert, H. S. (1998). Differential roles of homologous recombination pathways in *Neisseria gonorrhoeae* pilin antigenic variation, DNA transformation and DNA repair. *Mol Microbiol* **30**, 697-710.

Mercado-Blanco, J., van der Drift, K. M. G. M., Olsson, P. E., Thomas-Oates, J. E., van Loon, L. C. & Bakker, P. A. H. M. (2001). Analysis of the *pmsCEAB* gene cluster involved in biosynthesis of salicylic acid and the siderophore pseudomonine in the biocontrol strain *Pseudomonas fluorescens* WCS374. *J Bacteriol* **183**, 1909-1920.

Metzgar, D. & Wills, C. (2000). Evidence for the adaptive evolution of mutation rates. *Cell* **101**, 581-584.

Meyer, J. M., Neely, A., Stintzi, A., Georges, C. & Holder, I. A. (1996). Pyoverdin is essential for virulence of *Pseudomonas aeruginosa*. *Infect Immun* **64**, 518-523.

Meyer, J. M., Stintzi, A. & Poole, K. (1999). The ferripyoverdine receptor FpvA of *Pseudomonas aeruginosa* PAO1 recognizes the ferripyoverdines of *P. aeruginosa* PAO1 and *P. fluorescens* ATCC 13525. *FEMS Microbiol Lett* **170**, 145-150.

Meyer, J. M. (2000). Pyoverdines: pigments, siderophores and potential taxonomic markers of fluorescent *Pseudomonas* species. *Arch Microbiol* **174**, 135-142.

Meyer, T. F. & Hill, S. A. (2003). Genetic variation in the pathogenic *Neisseria* species. In *Antigenic Variation*, pp. 142-164. Edited by A. Craig & A. Scherf. London (UK): Elsevier Academic Press.

Miller, J. H. (1998). Mutators in *Escherichia coli*. *Mutat Res* **409**, 99-106.

Mirleau, P., Delorme, S., Philippot, L., Meyer, J. M., Mazurier, S. & Lemanceau, P. (2000). Fitness in soil and rhizosphere of *Pseudomonas fluorescens* C7R12 compared with a C7R12 mutant affected in pyoverdine synthesis and uptake. *FEMS Microbiol Ecol* **34**, 35-44.

Moenne-Loccoz, Y., Tichy, H. V., O'Donnell, A., Simon, R. & O'Gara, F. (2001). Impact of 2,4-diacetylphloroglucinol-producing biocontrol strain *Pseudomonas fluorescens* F113 on intraspecific diversity of resident culturable fluorescent pseudomonads associated with the roots of field-grown sugar beet seedlings. *Appl Environ Microbiol* **67**, 3418-3425.

Molina, M. A., Godoy, P., Ramos-Gonzalez, M. I., Munoz, N., Ramos, J. L. & Espinosa-Urgel, M. (2005). Role of iron and the TonB system in colonization of corn seeds and roots by *Pseudomonas putida* KT2440. *Environ Microbiol* **7**, 443-449.

Mossialos, D., Meyer, J. M., Budzikiewicz, H., Wolff, U., Koedam, N., Baysse, C., Anjaiah, V. L. & Cornelis, P. (2000). Quinolobactin, a new siderophore of *Pseudomonas fluorescens* ATCC 17400, the production of which is repressed by the cognate pyoverdine. *Appl Environ Microbiol* **66**, 487-492.

Moulin, L., Munive, A., Dreyfus, B. & Boivin-Masson, C. (2001). Nodulation of legumes by members of the β-subclass of Proteobacteria. *Nature* **411**, 948-950.

Moxon, E. R., Rainey, P. B., Nowak, M. A. & Lenski, R. E. (1994). Adaptive evolution of highly mutable loci in pathogenic bacteria. *Curr Biol* **4**, 24-33.

Naseby, D. C. & Lynch, J. M. (1999). Effects of *Pseudomonas fluorescens* F113 on ecological functions in the pea rhizosphere are dependent on pH. *Microb Ecol* **37**, 248-256.

Natsch, A., Keel, C., Pfirter, H. A., Haas, D. & Defago, G. (1994). Contribution of the global regulator gene *gacA* to persistence and dissemination of *Pseudomonas fluorescens* biocontrol strain CHAO introduced into soil microcosms. *Appl Environ Microbiol* **60**, 2553-2560.

Neilands, J. B. (1995). Siderophores: structure and function of microbial iron transport compounds. *J Biol Chem* **270**, 26723-26726.

Nielsen, T. H., Sorensen, D., Tobiasen, C., Andersen, J. B., Christophersen, C., Givskov, M. & Sorensen, J. (2002). Antibiotic and biosurfactant properties of cyclic lipopeptides produced by fluorescent *Pseudomonas* spp. from the sugar beet rhizosphere. *Appl Environ Microbiol* **68**, 3416-3423.

Notz, R., Maurhofer, M., Dubach, H., Haas, D. & Défago, G. (2002). Fusaric acid-producing strains of *Fusarium oxysporum* alter 2,4-diacetylphloroglucinol biosynthetic gene expression in *Pseudomonas fluorescens* CHA0 in vitro and in the rhizosphere of wheat. *Appl Environ Microbiol* **68**, 2229-2235.

Nou, X. W., Braaten, B., Kaltenbach, L. & Low, D. A. (1995). Differential binding of Lrp to two sets of *pap* DNA-binding sites mediated by PapI regulates Pap phase variation in *Escherichia coli*. *EMBO Journal* **14**, 5785-5797.

Nowak-Thompson, B., Chaney, N., Wing, J. S., Gould, S. J. & Loper, J. E. (1999). Characterization of the pyoluteorin biosynthetic gene cluster of *Pseudomonas fluorescens* Pf-5. *J Bacteriol* **181**, 2166-2174.

O'Toole, G. A. & Kolter, R. (1998). Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol* **30**, 295-304.

Ochsner, U. A., Johnson, Z. & Vasil, M. L. (2000). Genetics and regulation of two distinct haem-uptake systems, *phu* and *has*, in *Pseudomonas aeruginosa*. *Microbiol* **146**, 185-198.

Ojanen-Reuhs, T., Kalkkinen, N., Westerlund-Wikstrom, B., vanDoorn, J., Haahtela, K., Nurmiaho-Lassila, E. L., Wengelnik, K., Bonas, U. & Korhonen, T. K. (1997). Characterization of the *fimA* gene encoding bundle-forming fimbriae of the plant pathogen *Xanthomonas campestris* pv vesicatoria. *J Bacteriol* **179**, 1280-1290.

Okkon, Y., Boloemberg, G. V. & Lugtenberg, B. J. J. (1998). Biotechnology of biofertilization and phytostimulation. In *Agricultural Biotechnology*, pp. 327-349. Edited by A. Altman. New York: Marcel Dekker Inc.

Oliver, A., Canton, R., Campo, P., Baquero, F. & Blazquez, J. (2000). High frequency of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *Science* **288**, 1251-1253.

Oliver, A., Sanchez, J. M. & Blazquez, J. (2002). Characterization of the GO system of *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* **217**, 31-35.

Ongena, M., Jacques, P., Delfosse, P. & Thonart, P. (2001). Unusual traits of the pyoverdin-mediated iron acquisition system in *Pseudomonas putida* strain BTP1. *Biometals* **15**, 1-13.

Osbourn, A. E., Barber, C. E. & Daniels, M. J. (1987). Identification of plant-induced genes of the bacterial pathogen *Xanthomonas campestris* pathovar campestris using a promoter-probe plasmid. *EMBO Journal* **6**, 23-28.

Palleroni, N. J. (1992). Human and animal-pathogenic pseudomonads. In *The Prokaryotes, A Handbook on the Biology of Bacteria, Ecophysiology, Isolation, Identification and Applications*, pp. 3086-3103. Edited by A. Belows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer-Verlag.

Pandza, S., Baetens, M., Park, C. H., Au, T., Keyhan, M. & Matin, A. (2000). The G-protein FlhF has a role in polar flagellar placement and general stress response induction in *Pseudomonas putida*. *Mol Microbiol* **36**, 414-423.

Park, D. & Forst, S. (2006). Co-regulation of motility, exoenzyme and antibiotic production by the EnvZ-OmpR-FlhDC-FliA pathway in *Xenorhabdus nematophila*. *Mol Microbiol*, doi:10.1111/j.1365-2958.2006.05320.x.

Park, S. F., Purdy, D. & Leach, S. (2000). Localized reversible frameshift mutation in the *flhA* gene confers phase variability to flagellin gene expression in *Campylobacter coli*. *J Bacteriol* **182**, 207-210.

Patten, C. L. & Glick, B. R. (2002). Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. *Appl Environ Microbiol* **68**, 3795-3801.

Pessi, G. & Haas, D. (2000). Transcriptional control of the hydrogen cyanide biosynthetic genes *hcnABC* by the anaerobic regulator ANR and the quorum-sensing regulators LasR and RhlR in *Pseudomonas aeruginosa*. *J Bacteriol* **182**, 6940-6949.

Pessi, G., Williams, F., Hindle, Z., Heurlier, K., Holden, M. T. G., Camara, M., Haas, D. & Williams, P. (2001). The global posttranscriptional regulator RsmA modulates production of virulence determinants and *N*-acylhomoserine lactones in *Pseudomonas aeruginosa*. *J Bacteriol* **183**, 6676-6683.

Pfender, W. F., Kraus, J. & Loper, J. E. (1993). A genomic region from *Pseudomonas fluorescens* Pf-5 required for pyrrolnitrin production and inhibition of *Pyrenophora tritici-repentis* in wheat straw. *Phytopathology* **83**, 1223-1228.

Pierson, L. S., Keppenne, V. D. & Wood, D. W. (1994). Phenazine antibiotic biosynthesis in *Pseudomonas aureofaciens* 30-84 is regulated by PhzR in response to cell density. *J Bacteriol* **176**, 3966-3974.

Pieterse, C. M. J., van Wees, S. C. M., Hoffland, E., van Pelt, J. A. & van Loon, L. C. (1996). Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis related gene expression. *Plant Cell* **8**, 1225-1237.

Pieterse, C. M. J., van Wees, S. C. M., van Pelt, J. A., Knoester, M., Laan, R., Gerrits, N., Weisbeek, P. J. & van Loon, L. C. (1998). A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. *Plant Cell* **10**, 1571-1580.

Pieterse, C. M. J., Van Pelt, J. A., Ton, J., Parchmann, S., Mueller, M. J., Buchala, A. J., Metraux, J. P. & Van Loon, L. C. (2000). Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* requires sensitivity to jasmonate and ethylene but is not accompanied by an increase in their production. *Physiol Mol Plant Pathol* **57**, 123-134.

Prigent-Combaret, C., Vidal, O., Dorel, C. & Lejeune, P. (1999). Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli*. *J Bacteriol* **181**, 5993-6002.

Prouty, M. G., Correa, N. E. & Klose, K. E. (2001). The novel σ^{54}- and σ^{28}-dependent flagellar gene transcription hierarchy of *Vibrio cholerae*. *Mol Microbiol* **39**, 1595-1609.

Prüβ, B. M. & Matsumura, P. (1996). A regulator of the flagellar regulon of *Escherichia coli*, *flhD*, also affects cell division. *J Bacteriol* **178**, 668-674.

Quiñones, B., Dulla, G. & Lindow, S. E. (2005). Quorum sensing regulates exopolysaccharide production, motility, and virulence in *Pseudomonas syringae*. *Mol Plant-Microbe Interact* **18**, 682-693.

Raaijmakers, J. M., Vandersluis, I., Koster, M., Bakker, P. A. H. M., Weisbeek, P. J. & Schippers, B. (1995). Utilization of heterologous siderophores and rhizosphere competence of fluorescent *Pseudomonas* spp. *Can J Microbiol* **41**, 126-135.

Raaijmakers, J. M., de Bruijn, I. & de Kock, M. J. D. (2006). Cyclic lipopeptide production by plant-associated *Pseudomonas* spp.: diversity, activity, biosynthesis, and regulation. *Mol Plant-Microbe Interact* **19**, 699-710.

Rahme, L. G., Stevens, E. J., Wolfort, S. F., Shao, J., Tompkins, R. G. & Ausubel, F. M. (1995). Common virulence factors for bacterial pathogenicity in plants and animals. *Science* **268**, 1899-1902.

Rainey, P. B. & Travisano, M. (1998). Adaptive radiation in a heterogeneous environment. *Nature* **394**, 69-72.

Rainey, P. B. (1999). Adaptation of *Pseudomonas fluorescens* to the plant rhizosphere. *Environ Microbiol* **1**, 243-257.

Ramos-González, M. I., Campos, M. J. & Ramos, J. L. (2005). Analysis of *Pseudomonas putida* KT2440 gene expression in the maize rhizosphere: In vivo expression technology capture and identification of root-activated promoters. *J Bacteriol* **187**, 5504-5504.

Rasolomampianina, R., Bailly, X., Fetiarison, R. & other authors (2005). Nitrogen-fixing nodules from rose wood legume trees (*Dalbergia* spp.) endemic to Madagascar host seven different genera belonging to α- and β-Proteobacteria. *Mol Ecol* **14**, 4135-4146.

Redly, G. A. & Poole, K. (2003). Pyoverdine-mediated regulation of FpvA synthesis in *Pseudomonas aeruginosa*: involvement of a probable extracytoplasmic-function sigma factor, FpvI. *J Bacteriol* **185**, 1261-1265.

Reimmann, C., Beyeler, M., Latifi, A., Winteler, H., Foglino, M., Lazdunski, A. & Haas, D. (1997). The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer N-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. *Mol Microbiol* **24**, 309-319.

Reimmann, C., Valverde, C., Kay, E. & Haas, D. (2005). Posttranscriptional repression of GacS/GacA-controlled genes by the RNA-binding protein RsmE acting together with RsmA in biocontrol strain *Pseudomonas fluorescens* CHA0. *J Bacteriol* **187**, 276-285.

Reinhold-Hurek, B. & Hurek, T. (1998). Life in grasses: diazotrophic endophytes. *Trends Microbiol* **6**, 202-202.

Rich, J. J., Kinscherf, T. G., Kitten, T. & Willis, D. K. (1994). Genetic evidence that the *gacA* gene encodes the cognate response regulator for the *lemA* sensor in *Pseudomonas syringae*. *J Bacteriol* **176**, 7468-7475.

Richardson, A. R., Yu, Z., Popovic, T. & Stojiljkovic, I. (2002). Mutator clones of *Neisseria meningitidis* in epidemic serogroup A disease. *Proc Natl Acad Sci U S A* **99**, 6103-6107.

Ritchings, B. W., Almira, E. C., Lory, S. & Ramphal, R. (1995). Cloning and phenotypic characterization of *fleS* and *fleR*, new response regulators of *Pseudomonas aeruginosa* which regulate motility and adhesion to mucin. *Infect Immun* **63**, 4868-4876.

Robleto, E. A., López-Hernández, I., Silby, M. W. & Levy, S. B. (2003). Genetic analysis of the AdnA regulon in *Pseudomonas fluorescens*: nonessential role of flagella in adhesion to sand and biofilm formation. *J Bacteriol* **185**, 453-460.

Roine, E., Raineri, D. M., Romantschuk, M., Wilson, M. & Nunn, D. N. (1998). Characterization of type IV pilus genes in *Pseudomonas syringae* pv. *tomato* DC3000. *Mol Plant-Microbe Interact* **11**, 1048-1056.

Romeo, T. (1998). Global regulation by the small RNA-binding protein CsrA and the non-coding RNA molecule *csrB*. *Mol Microbiol* **29**, 1321-1330.

Rovira, A. D. (1956). A study of the development of the root surface microflora during the initial stages of plant growth. *J Appl Bacteriol* **19**, 72-79.

Sacherer, P., Defago, G. & Haas, D. (1994). Extracellular protease and phospholipase-C are controlled by the global regulatory gene *gacA* in the biocontrol strain *Pseudomonas fluorescens* CHA0. *FEMS Microbiol Lett* **116**, 155-160.

Sadowski, P. (1986). Site-specific recombinases: changing partners and doing the twist. *J Bacteriol* **165**, 341-347.

Saleh, S. S. & Glick, B. R. (2001). Involvement of *gacS* and *rpoS* in enhancement of the plant growth-promoting capabilities of *Enterobacter cloacae* CAL2 and UW4. *Can J Microbiol* **47**, 698-705.

Sánchez-Contreras, M. (2001). Variación de fase en *Pseudomonas fluorescens* F113 durante la colonización de la rizosfera de alfalfa. Madrid: Universidad Autónoma de Madrid.

Sánchez-Contreras, M., Martín, M., Villacieros, M., O'Gara, F., Bonilla, I. & Rivilla, R. (2002). Phenotypic selection and phase variation occur during alfalfa root colonization by *Pseudomonas fluorescens* F113. *J Bacteriol* **184**, 1587-1596.

Sarkari, J., Pandit, N., Moxon, E. R. & Achtman, M. (1994). Variable expression of the Opc outer membrane protein in *Neisseria meningitidis* is caused by size variation of a promoter containing poly-cytidine. *Mol Microbiol* **13**, 207-217.

Saumaa, S., Tover, A., Kasak, L. & Kivisaar, M. (2002). Different spectra of stationary-phase mutations in early-arising versus late-arising mutants of *Pseudomonas putida*: Involvement of the DNA repair enzyme MutY and the stationary-phase sigma factor RpoS. *J Bacteriol* **184**, 6957-6965.

Saunders, N. J., Peden, J. F., Hood, D. W. & Moxon, E. R. (1998). Simple sequence repeats in the *Helicobacter pylori* genome. *Mol Microbiol* **27**, 1091-1098.

Saunders, N. J., Moxon, E. R. & Gravenor, M. B. (2003). Mutation rates: estimating phase variation rates when fitness differences are present and their impact on population structure. *Microbiol* **149**, 485-495.

Scher, F. M. & Baker, R. (1982). Effects of *Pseudomonas putida* and a synthetic iron chelator on induction of soil suppressiveness to *Fusarium wilt* pathogens. *Phytopathology* **72**, 1567-1573.

Scher, F. M., Kloepper, J. W., Singleton, C., Zaleska, I. & Laliberte, M. (1988). Colonization of soybean roots by *Pseudomonas* and *Serratia* species: relationship to bacterial motility, chemotaxis, and generation time. *Phytopathology* **78**, 1055-1059.

Schippers, B., Bakker, A. W. & Bakker, P. A. H. M. (1987). Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. *Annu Rev Phytopathol* **25**, 339-358.

Schloter, M., Wiehe, W., Assmus, B., Steindl, H., Becke, H., Hoflich, G. & Hartmann, A. (1997). Root colonization of different plants by plant-growth-promoting *Rhizobium leguminosarum* bv. trifolii R39 studied with monospecific polyclonal antisera. *Appl Environ Microbiol* **63**, 2038-2046.

Schmidli-Sacherer, P., Keel, C. & Défago, G. (1997). The global regulator GacA of *Pseudomonas fluorescens* CHA0 is required for suppression of root diseases in dicotyledons but not in gramineae. *Plant Pathology* **46**, 80-90.

Schnider-Keel, U., Seematter, A., Maurhofer, M. & other authors (2000). Autoinduction of 2,4-diacetylphloroglucinol biosynthesis in the biocontrol agent *Pseudomonas fluorescens* CHA0 and repression by the bacterial metabolites salicylate and pyoluteorin. *J Bacteriol* **182**, 1215-1225.

Schofield, M. J. & Hsieh, P. (2003). DNA mismatch repair: molecular mechanisms and biological function. *Annu Rev Microbiol* **57**, 579-608.

Schroth, M., Hildebrand, D. C. & Panapoulos, N. (1992). Phytopathogenic pseudomonads and plant-associated pseudomonads. In *The Prokaryotes, A Handbook on the Biology of Bacteria, Ecophysiology, Isolation, Identification and Applications*, pp. 3104-3131. Edited by A. Belows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer-Verlag.

Segura, A., Hurtado, A., Duque, E. & Ramos, J. L. (2004). Transcriptional phase variation at the *flhB* gene of *Pseudomonas putida* DOT-T1E is involved in response to environmental changes and suggests the participation of the flagellar export system in solvent tolerance. *J Bacteriol* **186**, 1905-1909.

Seifert, H. S. (1996). Questions about gonococcal pilus phase and antigenic variation. *Mol Microbiol* **21**, 433-440.

Sexton, R., Gill, P. R., Dowling, D. N. & O´Gara, F. (1996). Transcriptional regulation of the iron-responsive sigma factor gene *pbrA*. *Mol Gen Genet* **250**, 50-58.

Shanahan, P., O´Sullivan, D. J., Simpson, P., Glennon, J. D. & O´Gara, F. (1992). Isolation of 2,4-diacetylphloroglucinol from a fluorescent pseudomonad and investigation of physiological parameters influencing its production. *Appl Environ Microbiol* **58**, 353-358.

Shapiro, J. A. (1997). Genome organization, natural genetic engineering and adaptive mutation. *Trends Genet* **13**, 98-104.

Shevchik, V. E., Bortoligerman, I., Robertbaudouy, J., Robinet, S., Barras, F. & Condemine, G. (1995). Differential effect of *dsbA* and *dsbC* mutations on extracellular enzyme secretion in *Erwinia chrysanthemi*. *Mol Microbiol* **16**, 745-753.

Shimada, T., Sakazaki, R. & Suzuki, K. (1985). Peritrichous flagella in mesophilic strains of *Aeromonas*. *Jpn J Med Sci Biol* **38**, 141-145.

Shin, S. & Park, C. (1995). Modulation of flagellar expression in *Escherichia coli* by acetyl phosphate and the osmoregulator OmpR. *J Bacteriol* **177**, 4696-4702.

Silby, M. W. & Levy, S. B. (2004). Use of in vivo expression technology to identify genes important in growth and survival of *Pseudomonas fluorescens* Pf0-1 in soil: Discovery of expressed sequences with novel genetic organization. *J Bacteriol* **186**, 7411-7419.

Simons, M., van der Bij, A. J., Brand, I., de Weger, L. A., Wijffelman, C. A. & Lugtenberg, B. J. J. (1996). Gnotobiotic system for studying rhizosphere colonization by plant growth-promoting *Pseudomonas* bacteria. *Mol Plant-Microbe Interact* **9**, 600-607.

Simons, M., Permentier, H. P., de Weger, L. A., Wijffelman, C. A. & Lugtenberg, B. J. J. (1997). Amino acid synthesis is necessary for tomato root colonization by *Pseudomonas fluorescens* strain WCS365. *Mol Plant-Microbe Interact* **10**, 102-106.

Sinha, H., Pain, A. & Johnstone, K. (2000). Analysis of the role of *recA* in phenotypic switching of *Pseudomonas tolaasii*. *J Bacteriol* **182**, 6532-6535.

Soutourina, O., Kolb, A., Krin, E., Laurent-Winter, C., Rimsky, S., Danchin, A. & Bertin, P. (1999). Multiple control of flagellum biosynthesis in *Escherichia coli*: role of H-NS protein and the cyclic AMP-catabolite activator protein complex in transcription of the *flhDC* master operon. *J Bacteriol* **181**, 7500-7508.

Spangenberg, C., Heuer, T., Burger, C. & Tummler, B. (1996). Genetic diversity of flagellins of *Pseudomonas aeruginosa*. *FEBS Lett* **396**, 213-217.

Sperandio, V., Torres, A. G. & Kaper, J. B. (2002). Quorum sensing *Escherichia coli* regulators B and C (QseBC): a novel two-component regulatory system involved in the regulation of flagella and motility by quorum sensing in *E. coli*. *Mol Microbiol* **43**, 809-821.

Starnbach, M. N. & Lory, S. (1992). The *fliA* (*rpoF*) gene of *Pseudomonas aeruginosa* encodes an alternative sigma factor required for flagellin synthesis. *Mol Microbiol* **6**, 459-469.

Steenhoudt, O. & Vanderleyden, J. (2000). *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. *FEMS Microbiol Rev* **24**, 487-506.

Stentebjerg-Olesen, B., Chakraborty, T. & Klemm, P. (2000). FimE-catalyzed off-to-on inversion of the type 1 fimbrial phase switch and insertion sequence recruitment in an *Escherichia coli* K-12 *fimB* strain. *FEMS Microbiol Lett* **182**, 319-325.

Stern, A., Brown, M., Nickel, P. & Meyer, T. F. (1986). Opacity genes in *Neisseria gonorrhoeae*: control of phase and antigenic variation. *Cell* **47**, 61-71.

Stibitz, S., Aaronson, W., Monack, D. & Falkow, S. (1989). Phase variation in *Bordetella pertussis* by frameshift mutation in a gene for a novel two-component system. *Nature* **338**, 266-269.

Stintzi, A., Evans, K., Meyer, J. M. & Poole, K. (1998). Quorum-sensing and siderophore biosynthesis in *Pseudomonas aeruginosa*: *lasR/lasI* mutants exhibit reduced pyoverdine biosynthesis. *FEMS Microbiol Lett* **166**, 341-345.

Stolworthy, J. C., Paszczynski, A., Korus, R. & Crawford, R. L. (2001). Metal binding by pyridine-2,6-bis(monothiocarboxylic acid), a biochelator produced by *Pseudomonas stutzeri* and *Pseudomonas putida*. *Biodegradation* **12**, 411-418.

Streit, W. R. & Phillips, D. A. (1997). A biotin-regulated locus, *bioS*, in a possible survival operon of *Rhizobium meliloti*. *Mol Plant-Microbe Interact* **10**, 933-937.

Suh, S. J., Silo-Suh, L., Woods, D. E., Hassett, D. J., West, S. E. H. & Ohman, D. E. (1999). Effect of *rpoS* mutation on the stress response and expression of virulence factors in *Pseudomonas aeruginosa*. *J Bacteriol* **181**, 3890-3897.

Suzuki, K., Wang, X., Weilbacher, T., Pernestig, A. K., Melefors, O., Georgellis, D., Babitzke, P. & Romeo, T. (2002). Regulatory circuitry of the CsrA/*csrB* and BarA/UvrY systems of *Escherichia coli*. *J Bacteriol* **184**, 5130-5140.

Taddei, F., Radman, M., MaynardSmith, J., Toupance, B., Gouyon, P. H. & Godelle, B. (1997). Role of mutator alleles in adaptive evolution. *Nature* **387**, 700-702.

Takase, H., Nitanai, H., Hoshino, K. & Otani, T. (2000). Impact of siderophore production on *Pseudomonas aeruginosa* infections in immunosuppressed mice. *Infect Immun* **68**, 1834-1839.

Tan, M. W., Rahme, L. G., Sternberg, J. A., Tompkins, R. G. & Ausubel, F. M. (1999). *Pseudomonas aeruginosa* killing of *Caenorhabditis elegans* used to identify *P .aeruginosa* virulence factors. *Proc Natl Acad Sci U S A* **96**, 2408-2413.

Tart, A. H., Wolfgang, M. C. & Wozniak, D. J. (2005). The alternative sigma factor AlgT represses *Pseudomonas aeruginosa* flagellum biosynthesis by inhibiting expression of *fleQ*. *J Bacteriol* **187**, 7955-7962.

Tegova, R., Tover, A., Tarassova, K., Tark, M. & Kivisaar, M. (2004). Involvement of error-prone DNA polymerase IV in stationary-phase mutagenesis in *Pseudomonas putida*. *J Bacteriol* **186**, 2735-2744.

Thomashow, L. S. & Weller, D. M. (1988). Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. tritici. *J Bacteriol* **170**, 3499-3508.

Tomenius, H., Pernestig, A. K., Jonas, K., Georgellis, D., Mollby, R., Normark, S. & Melefors, O. (2006). The *Escherichia coli* BarA-UvrY two-component system is a virulence determinant in the urinary tract. *BMC Microbiol* **6**, 27.

Ton, J., Van Pelt, J. A., Van Loon, L. C. & Pieterse, C. M. J. (2002). Differential effectiveness of salicylate-dependent and jasmonate/ethylene-dependent induced resistance in *Arabidopsis*. *Mol Plant-Microbe Interact* **15**, 27-34.

Touati, D. (2000). Iron and oxidative stress in bacteria. *Arch Biochem Biophys* **373**, 1-6.

Valverde, C., Lindell, M., Wagner, E. G. H. & Haas, D. (2004). A repeated GGA motif is critical for the activity and stability of the riboregulator *rsmY* of *Pseudomonas fluorescens*. *J Biol Chem* **279**, 25066-25074.

van den Broek, D., Chin-A-Woeng, T. F. C., Eijkemans, K., Mulders, I. H. M., Bloemberg, G. V. & Lugtenberg, B. J. J. (2003). Biocontrol traits of *Pseudomonas* spp. are regulated by phase variation. *Mol Plant-Microbe Interact* **16**, 1003-1012.

van den Broek, D., Bloemberg, G. V. & Lugtenberg, B. (2005a). The role of phenotypic variation in rhizosphere *Pseudomonas* bacteria. *Environ Microbiol* **7**, 1686-1697.

van den Broek, D., Chin-A-Woeng, T. F. C., Bloemberg, G. V. & Lugtenberg, B. J. J. (2005b). Molecular nature of spontaneous modifications in *gacS* which cause colony phase variation in *Pseudomonas* sp strain PCL1171. *J Bacteriol* **187**, 593-600.

van der Woude, M., Braaten, B. & Low, D. (1996). Epigenetic phase variation of the *pap* operon in *Escherichia coli*. *Trends Microbiol* **4**, 5-9.

van Loon, L. C., Bakker, P. A. H. M. & Pieterse, C. M. J. (1998). Systemic resistance induced by rhizosphere bacteria. *Annu Rev Phytopathol* **36**, 453-483.

Van Overbeek, L. S. & Van Elsas, J. D. (1995). Root exudate-induced promoter activity in *Pseudomonas fluorescens* mutants in the wheat rhizosphere. *Appl Environ Microbiol* **61**, 890-898.

van Rij, E. T., Wesselink, M., Chin-A-Woeng, T. F. C., Bloemberg, G. V. & Lugtenberg, B. J. J. (2004). Influence of environmental conditions on the production of phenazine-1-carboxamide by *Pseudomonas chlororaphis* PCL1391. *Mol Plant-Microbe Interact* **17**, 557-566.

van Rij, E. T., Girard, G., Lugtenberg, B. J. J. & Bloemberg, G. V. (2005). Influence of fusaric acid on phenazine-1-carboxamide synthesis and gene expression of *Pseudomonas chlororaphis* strain PCL1391. *Microbiol* **151**, 2805-2814.

Van Wees, S. C. M., Pieterse, C. M. J., Trijssenaar, A., Vant Westende, Y. A. M., Hartog, F. & Van Loon, L. C. (1997). Differential induction of systemic resistance in *Arabidopsis* by biocontrol bacteria. *Mol Plant-Microbe Interact* **10**, 716-724.

Vande Broek, A. V. & Vanderleyden, J. (1995). The role of bacterial motility, chemotaxis, and attachment in bacteria plant interactions. *Mol Plant-Microbe Interact* **8**, 800-810.

Vander Wauven, C., Pierard, A., Kleyraymann, M. & Haas, D. (1984). *Pseudomonas aeruginosa* mutants affected in anaerobic growth on arginine: evidence for a four-gene cluster encoding the arginine deiminase pathway. *J Bacteriol* **160**, 928-934.

Ventre, I., Goodman, A. L., Vallet-Gely, I. & other authors (2006). Multiple sensors control reciprocal expression of *Pseudomonas aeruginosa* regulatory RNA and virulence genes. *Proc Natl Acad Sci U S A* **103**, 171-176.

Villacieros, M. (2000). Diseño de un sistema integrado planta/microorganismo para su uso en biorremediación. Madrid: Universidad Autónoma de Madrid.

Villacieros, M., Power, B., Sánchez-Contreras, M. & other authors (2003). Colonization behaviour of *Pseudomonas fluorescens* and *Sinorhizobium meliloti* in the alfalfa (*Medicago sativa*) rhizosphere. *Plant Soil* **251**, 47-54.

Villacieros, M., Whelan, C., Mackova, M. & other authors (2005). Polychlorinated biphenyl rhizoremediation by *Pseudomonas fluorescens* F113 derivatives, using a *Sinorhizobium meliloti* nod system to drive *bph* gene expression. *Appl Environ Microbiol* **71**, 2687-2694.

Visca, P., Leoni, L., Wilson, M. J. & Lamont, I. L. (2002). Iron transport and regulation, cell signalling and genomics: lessons from *Escherichia coli* and *Pseudomonas*. *Mol Microbiol* **45**, 1177-1190.

Waite, R. D., Penfold, D. W., Struthers, J. K. & Dowson, C. G. (2003). Spontaneous sequence duplications within capsule genes *cap8E* and *tts* control phase variation in *Streptococcus pneumoniae* serotypes 8 and 37. *Microbiol* **149**, 497-504.

Watnick, P. I., Lauriano, C. M., Klose, K. E., Croal, L. & Kolter, R. (2001). The absence of a flagellum leads to altered colony morphology, biofilm development and virulence in *Vibrio cholerae* O139. *Mol Microbiol* **39**, 223-235.

Wei, B. D. L., Brun-Zinkernagel, A. M., Simecka, J. W., Pruss, B. M., Babitzke, P. & Romeo, T. (2001). Positive regulation of motility and *flhDC* expression by the RNA-binding protein CsrA of *Escherichia coli*. *Mol Microbiol* **40**, 245-256.

Weilbacher, T., Suzuki, K., Dubey, A. K. & other authors (2003). A novel sRNA component of the carbon storage regulatory system of *Escherichia coli*. *Mol Microbiol* **48**, 657-670.

Weiser, J. N., Goldberg, J. B., Pan, N., Wilson, L. & Virji, M. (1998). The phosphorylcholine epitope undergoes phase variation on a 43-Kilodalton protein in *Pseudomonas aeruginosa* and on pili of *Neisseria meningitidis* and *Neisseria gonorrhoeae*. *Infect Immun* **66**, 4263-4267.

Welbaum, G. E., Sturz, A. V., Dong, Z. M. & Nowak, J. (2004). Managing soil microorganisms to improve productivity of agro-ecosystems. *Crit Rev Plant Sci* **23**, 175-193.

Whistler, C. A., Corbell, N. A., Sarniguet, A., Ream, W. & Loper, J. E. (1998). The two-component regulators GacS and GacA influence accumulation of the stationary-phase sigma factor σ^s and the stress response in *Pseudomonas fluorescens* Pf-5. *J Bacteriol* **180**, 6635-6641.

Wilson, M. J., McMorran, B. J. & Lamont, I. L. (2001). Analysis of promoters recognized by PvdS, an extracytoplasmic-function sigma factor protein from *Pseudomonas aeruginosa*. *J Bacteriol* **183**, 2151-2155.

Willis, D. K., Holmstadt, J. J. & Kinscherf, T. G. (2001). Genetic evidence that loss of virulence associated with *gacS* or *gacA* mutations in *Pseudomonas syringae* B728a does not result from effects on alginate production. *Appl Environ Microbiol* **67**, 1400-1403.

Winslow, C., Broadhurst, J., Buchanan, R. E., Krumwiede, J. C., Rogers, L. A. & Smith, G. H. (1917). The families and genera of bacteria. *J Bacteriol* **2**, 505-566.

Wong, S. M., Carroll, P. A., Rahme, L. G., Ausubel, F. M. & Calderwood, S. B. (1998). Modulation of expression of the ToxR regulon in *Vibrio cholerae* by a member of the two-component family of response regulators. *Infect Immun* **66**, 5854-5861.

Wood, D. W. & Pierson, L. S. (1996). The *phzI* gene of *Pseudomonas aureofaciens* 30-84 is responsible for the production of a diffusible signal required for phenazine antibiotic production. *Gene* **168**, 49-53.

Yang, Y. C., Chou, C. P., Kuo, T. T., Lin, S. H. & Yang, M. K. (2004). PilR enhances the sensitivity of *Xanthomonas axonopodis* pv. citri to the infection of filamentous bacteriophage Cf. *Curr Microbiol* **48**, 251-261.

Yao, J. & Allen, C. (2006). Chemotaxis is required for virulence and competitive fitness of the bacterial wilt pathogen *Ralstonia solanacearum*. *J Bacteriol* **188**, 3697-3708.

Ye, R. W., Haas, D., Ka, J. O., Krishnapillai, V., Zimmermann, A., Baird, C. & Tiedje, J. M. (1995). Anaerobic activation of the entire denitrification pathway in *Pseudomonas aeruginosa* requires ANR, an analog of FNR. *J Bacteriol* **177**, 3606-3609.

Yeiser, B., Pepper, E. D., Goodman, M. F. & Finkel, S. E. (2002). SOS-induced DNA polymerases enhance long-term survival and evolutionary fitness. *Proc Natl Acad Sci U S A* **99**, 8737-8741.

Yu, J., Peñaloza-Vazquez, A., Chakrabarty, A. M. & Bender, C. L. (1999). Involvement of the exopolysaccharide alginate in the virulence and epiphytic fitness of *Pseudomonas syringae* pv. *syringae*. *Mol Microbiol* **33**, 712-720.

Zieg, J., Silverman, M., Hilmen, M. & Simon, M. (1977). Recombinational switch for gene expression. *Science* **196**, 170-172.

Zuber, S., Carruthers, F., Keel, C. & other authors (2003). GacS sensor domains pertinent to the regulation of exoproduct formation and to the biocontrol potential of *Pseudomonas fluorescens* CHA0. *Mol Plant-Microbe Interact* **16**, 634-644.

OBJETIVOS

La colonización de la rizosfera por microorganismos del suelo es un proceso muy importante y complejo, de gran interés para la utilización de microorganismos como inoculantes en tecnologías agrícolas y medioambientales. Dentro de estos microorganismos las *Pseudomonas* son uno de los más eficaces colonizadores de la rizosfera de las plantas, y actualmente, están siendo muy utilizadas como microorganismo modelo en estudios de colonización.

Pseudomonas fluorescens F113 es una estirpe que es capaz de colonizar muy diversos tipos de plantas (remolacha, tomate, guisante, sauce, alfalfa…) y que se ha mostrado efectiva frente a la enfermedad del "black-root-rot" del tabaco y del "damping off" producida por el hongo *Pitium ultimum* en la remolacha ya que produce DAPG, un compuesto antifúngico de amplio espectro. Además, se han construido derivados de esta bacteria que contienen los genes *bph* bajo el control de diferentes sistemas de regulación, que permiten la degradación de bifenilos policlorados y se plantea su uso en rizorremediación.

Por todas estas características hemos considerado oportuno centrarnos en el estudio de dos de los mecanismos más importantes que intervienen en la colonización de la rizosfera (movilidad y variación de fase) y así poder mejorar su capacidad colonizadora, que redundará en un mejor uso de esta estirpe como agente de biocontrol y rizorremediador. Los principales objetivos propuestos para este trabajo son:

1.- Estudiar la regulación de la síntesis del filamento flagelar y el movimiento en *Pseudomonas fluorescens* F113 y su relación con la colonización de la rizosfera.

2.- Estudiar la implicación de las recombinasas específicas de sitio (*sss y xerD*) en la aparición de los variantes de fase y su papel en la colonización de la rizosfera.

3.- Estudiar con mayor profundidad el genotipo y fenotipo de los variantes de fase. Obtención de variantes de fase más competitivos que la cepa silvestre.

CAPÍTULO 2

Analysis of *Pseudomonas fluorescens* F113 genes implicated in flagellar filament synthesis and their role in competitive root colonization

ABSTRACT

The ability of plant-associated micro-organisms to colonize and compete in the rhizosphere is specially relevant for the biotechnological application of micro-organisms as inoculants. Pseudomonads are one of the best root colonizers and they are widely used in plant-pathogen biocontrol and in soil bioremediation. This study analyses the motility mechanism of the well-known biocontrol strain *Pseudomonas fluorescens* F113. A 6.5 kb region involved in the flagellar filament synthesis, containing the *fliC*, *flaG*, *fliD*, *fliS*, *fliT* and *fleQ* genes and part of the *fleS* gene, was sequenced and mutants in this region were made. Several non-motile mutants affected in the *fliC*, *fliS* and *fleQ* genes, and a *fliT* mutant with reduced motility properties, were obtained. These mutants were completely displaced from the root tip when competing with the wild-type F113 strain, indicating that the wild-type motility properties are necessary for competitive root colonization. A mutant affected in the *flaG* gene had longer flagella, but the same motility and colonization properties as the wild-type. However, in rich medium or in the absence of iron limitation, it showed a higher motility, suggesting the possibility of improving competitive root colonization by manipulating the motility processes.

INTRODUCTION

The study of rhizosphere colonization by micro-organisms is crucial for the efficient application of bacteria as inoculants, both in agricultural and in environmental biotechnology processes. *Pseudomonas* spp. can colonize the roots of a wide range of plants (Naseby & Lynch, 1998; Simons *et al.*, 1996; Villacieros *et al.*, 2003), being one of the best root colonizers, and are used as a model in root-colonization studies (Bloemberg *et al.*, 2000; Chin-A-Woeng *et al.*, 2000). The rhizosphere is a complex environment that supports a large and metabolically active microbial population, several orders of magnitude higher than the non-rhizospheric soil. Many bacterial genes and traits have been shown to be involved in plant-root colonization (Lugtenberg & Dekkers, 1999; Lugtenberg *et al.*, 2001; Rainey, 1999). However, not only colonization but also the pseudomonads' ability to compete with the indigenous microbial population are essential to improve their biotechnological applications in the rhizosphere environment.

The soil-borne fluorescent pseudomonads are used as biocontrol inoculants because of their ability to produce some antifungal metabolites (Dowling & O´Gara, 1994; Walsh *et al.*, 2001). Other applications of pseudomonads include soil biofertilization and rhizoremediation (Brazil *et al.*, 1995; Höflich *et al.*, 1995; Ramos *et al.*, 1991; Yee *et al.*, 1998).

The strain *Pseudomonas fluorescens* F113 was isolated from the sugarbeet rhizosphere and it is used as a biocontrol agent against the fungal pathogen *Pythium ultimum*, which causes damping-off disease in sugarbeet seedlings. The biocontrol abilities of this strain are due mainly to the production of the antifungal metabolite DAPG (2,4-diacetylphloroglucinol) (Shanahan *et al.*, 1992). *P. fluorescens* F113 has also been genetically modified, by introducing the *bph* genes that encode the biphenyl degradative pathway, to be used in rhizoremediation of polychlorinated biphenyls (Brazil *et al.*, 1995; Karlson *et al.*, 1998). The efficacy of *P. fluorescens*

F113 as inoculant clearly depends on its capacity to compete and efficiently colonize the rhizosphere.

Motility seems to be very important in colonization since non-motile mutants of different *P. fluorescens* strains are severely affected in the root colonization. The defect was larger at sites more distant from the inoculation site, in the root systems formed after the bacterial inoculation (Chin-A-Woeng *et al.*, 2000; de Weger *et al.*, 1987; Dekkers *et al.*, 1998b). Furthermore motility-impaired mutants of *Pseudomonas chlororaphis* PCL1391 do not reduce the disease produced by *Fusarium oxysporum* on tomato plants (Chin-A-Woeng *et al.*, 2000). Therefore, motility is required to colonize growing roots successfully and to maintain the biocontrol capacities.

The objective of this work was to study the phenotype of mutations affecting the flagellar filament synthesis in *P. fluorescens* F113 and their influence on motility and root competitive colonization. Reports to date refer to mutants that are either aflagellate (de Weger *et al.*, 1987) or deficient in chemotaxis (de Weert *et al.*, 2002). The mutants obtained in this work are not affected in chemotaxis but they are affected in motility to different degrees. We show that wild-type motility properties are necessary for competitive rhizosphere colonization.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this study are described in Table 2.1. *P. fluorescens* F113 was originally isolated from the sugarbeet rhizosphere (Shanahan *et al.*, 1992). The F113 gene bank was constructed with partially EcoRI-digested genomic DNA cloned into plasmid pLAFR3 in *Escherichia coli* LE392. Pseudomonas strains were grown on SA medium (Scher & Baker, 1982) at 28 ºC;

solid growth media contained 1.5% (w/v) purified agar. When appropriate, kanamycin, gentamicin and rifampicin were supplemented for antibiotic selection to

Table 2.1. Strains and plasmids used in this study

Strains / plasmids	Description	Reference or source
Strains		
DH5α	General purpose *Escherichia coli* strain	Gibco-BRL
F113rif	*Pseudomonas fluorescens* wild-type strain, Rif^r	(Shanahan *et al.*, 1992)
F113-fliC	F113rif fliC mutant, Rif^r, Km^r	This work
F113-flaG	F113rif flaG mutant, Rif^r, Km^r	This work
F113-fliS	F113rif fliS mutant, Rif^r, Km^r	This work
F113-fliT	F113rif fliT mutant, Rif^r, Km^r	This work
F113-fleQ	F113rif fleQ mutant, Rif^r, Km^r	This work
F113 (pBG1364)	F113rif containing plasmid with *fliC* under the control of the *nptII* promoter, Rif^r, Km^r, Gm^r	This work
F113 (pBG1308)	F113rif containing plasmid with *fliS* under the control of the *nptII* promoter, Rif^r, Km^r, Gm^r	This work
F113 (pBG1307)	F113rif containing plasmid with *fliT* under the control of the *nptII* promoter, Rif^r, Km^r, Gm^r	This work
F113 (pBG1259)	F113rif containing plasmid with *fleQ* under the control of the *nptII* promoter, Rif^r, Km^r, Gm^r	This work
Plasmids		
pVIK112	Suicide vector, Km^r	(Kalogeraki & Winans, 1997)
pRK2013	Helper plasmid used in triparental matings, Km^r	(Figurski & Helinski, 1979)
pML122	RSF1010 derivative expression vector, Gm^r, Km^r	(Labes *et al.*, 1990)
pBG1327	pVIK112 with an internal fragment of *fliC* used to generate mutant F113-fliC, Km^r	This work
pBG1342	pVIK112 with an internal fragment of *flaG* used to generate mutant F113-flaG, Km^r	This work
pBG1291	pVIK112 with an internal fragment of *fliS* used to generate mutant F113-fliS, Km^r	This work
pBG1339	pVIK112 with an internal fragment of *fliT* used to generate mutant F113-fliT, Km^r	This work
pBG1290	pVIK112 with an internal fragment of *fleQ* used to generate mutant F113-fleQ, Km^r	This work

a final concentration of 50, 10 and 100 μg/ml, respectively. *E. coli* strains were grown at 37 ºC in Luria–Bertani (LB) medium (Bertani, 1951), and antibiotics were added at the following concentrations when required: kanamycin, 25 μg/ml; gentamicin, 10 μg/ml.

DNA techniques

Standard techniques for subcloning procedures, plasmid preparations and agarose gel electrophoresis were used as described by Sambrook *et al*. (1989). Southern blot hybridizations were performed with a non-radioactive detection kit, and a chemiluminescence method was used to detect hybridization bands according to the manufacturer's instructions (Roche Diagnostics). DNA sequencing was done by the chain-termination method using DyeDeoxy terminator cycle sequencing kit protocol as described by the manufacturer (Applied Biosystems). Homology search and sequence analysis were done using the software from the Genetics Computer Group.

Construction of mutants

Insertional mutagenesis has been used to generate mutants by single homologous recombination. Amplified internal fragments from the different flagellar filament synthesis genes were cloned into the kanamycin-resistant plasmid pVIK112 (Kalogeraki & Winans, 1997) and introduced into wild-type F113 by triparental mating using pRK2013 as the helper plasmid (Figurski & Helinski, 1979). Mutants resulting from single homologous recombination were checked by Southern blotting using probes from the interrupted genes, and by PCR using primers designed from the genes and the pVIK112 plasmid sequences (the primer sequences are available on request). Mutant complementation analysis was done by cloning each intact gene under the control of the *nptII* strong promoter into plasmid pML122 (Labes *et al.*, 1990) and introducing the recombinant plasmid into the

corresponding mutant strain by triparental mating. Then, in order to correlate the strain phenotype with the interrupted gene, disappearance of the mutant phenotype was analysed.

Transmission electron microscopy

Formvar-coated grids were placed on the top of a drop of bacterial cells for 30 s to allow bacterial adhesion. Grids were stained for 1 min with a 1% solution of potassium phosphotungstate and washed for 1 min with a drop of water. Flagellum length was measured with the Q-Win software (Leica).

Swimming assays

SA (Scher & Baker, 1982), LB (Bertani, 1951) and iron-supplemented SA medium plates containing 0.3% purified agar were used to test the swimming abilities of wild-type F113 and the different mutants. The cells were inoculated in the middle of the plate, in triplicate, using a toothpick, from exponentially growing cultures. Swimming haloes were measured after 18, 24 and 42 h inoculation. Every assay was done at least three times.

Colonization experiments

Alfalfa seeds were sterilized in 70% ethanol for 2 min and in diluted bleach (1:5, v/v) for 15 min and rinsed thoroughly with sterile distilled water. Seeds were germinated at 4 ºC for 16 h followed by incubation in darkness, at 28 ºC for 1 day. Germinated alfalfa seeds were sown in Leonard jar gnotobiotic systems using Perlite as the solid substrate and 8 mM KNO_3-supplemented FP (Fahraeus, 1957) as the mineral solution. After 2 days, alfalfa seedlings were inoculated with ~10^8 cells of the appropriate strain. For the competitive colonization experiments, the tested strain and the competitor were inoculated at a ratio of 1:1. Plants were maintained

for 3 weeks in a plant growth cabinet in the following controlled conditions: 16 h of light at 25 ºC and 8 h of dark at 18 ºC. Bacteria were recovered from the last centimetre of the main root by vortexing for 2 min in 5 ml of 0.9% NaCl and appropriate dilutions were plated in SA supplemented with the selective antibiotics. The mean of recovered bacteria per g of root tip was 2.48 x 10^{7}, the range being from 1.35 x 10^{6} to 2.96 x 10^{8}. Colonization experiments were done three times in triplicate with at least 20 plants per replica.

Protein extraction and Western blots

Proteins were extracted from 200 ml cultures grown for 2 days. In order to detach the flagellar filaments, the cultures were agitated by vortexing for 2 min and then centrifuged for 20 min at 12 000 r.p.m. Total proteins were extracted from the pellet with Laemmli buffer (Laemmli, 1970) and extracellular proteins were extracted from the supernatant, by precipitation for 16 h at 4 ºC with 10% (w/v) TCA, followed by two washes with acetone, and were finally resuspended in Laemmli buffer. Proteins were electrophoresed in 12% acrylamide gels and stained with Coomassie blue. The same electrophoretic conditions were used for Western blotting. Gels were transferred to nitrocellulose membranes and incubated with 1:10000 dilution of an anti-flagellin antiserum (Dekkers *et al.*, 1998a) and with a peroxidase-tagged secondary antibody (anti-rabbit immunoglobulin). In the dot-blot experiments, the culture was agitated by vortexing and was centrifuged to separate the flagellar filaments. A drop from the pellets obtained was transferred to the nitrocellulose membrane and incubated with the anti-flagellin antiserum in the same conditions as described above.

RESULTS

Characterization of a genetic region containing genes involved in flagellar filament synthesis

Fig. 2.1 shows the genetic organization of a 6.5 kb DNA region from the *P. fluorescens* F113 genome that was isolated from a cosmid that contained the *fliC* gene, from an F113 gene bank (Sánchez-Contreras *et al.*, 2002). This region also contains another five ORFs and a partial ORF.

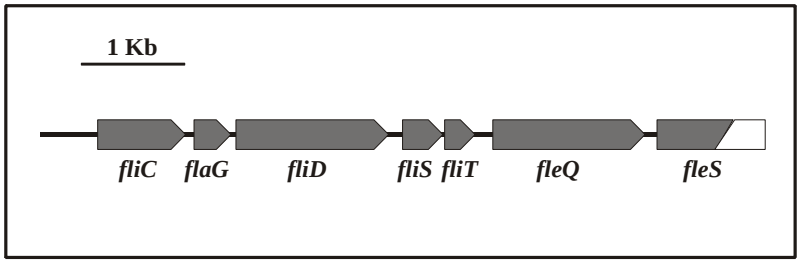

Figure 2.1. Physical map of the 6.5 Kb DNA region containing the genes implicated in *P. fluorescens* F113 flagellar filament synthesis.

Two of these complete ORFs and the partial ORF show high homology and synteny with the previously described genes *fliD*, encoding the filament cap protein, *fleQ* (*adnA*), encoding a master transcriptional regulator, and *fleS*, encoding a two component sensor protein, respectively. These genes have been found in every pseudomonad analysed (Arora *et al.*, 2000; Dasgupta *et al.*, 2002; Robleto *et al.*, 2003) and have been thoroughly characterized.

Downstream of the *fliC* gene, there is a small ORF homologous to ORFs with the same gene context in other pseudomonads. This ORF shows homology

with the *flaG* gene of *Vibrio anguillarum* (McGee *et al.*, 1996), which affects filament length through an unknown mechanism.

Table 2.2. FliT protein sequence comparison between *P. fluorescens* F113 and other bacteria

Strain	Identity (%)	Length (aa)*
Pseudomomas fluorescens Pf0-1	83	98
Pseudomonas syringae bv. tomato	69	98
Pseudomonas putida KT2440	56	97
Pseudomonas aeruginosa DG1	46	111
Pseudomonas aeruginosa PAO1	40	98
Pseudomonas aeruginosa PAK	37	96
Salmonella typhimurium	23	122
Escherichia coli	18	121

* In *P. fluorescens* F113 the FliT predicted protein is 98 aa in length.

Downstream of the *fliD* gene and after an AT-rich intergenic region there are two other small ORFs that show limited homology with the PA1095 and PA1096 genes in the *Pseudomonas aeruginosa* PAO1 genome (Stover *et al.*, 2000). The first ORF sequence shows high homology (77–79% identity) with the FliS protein in *P. fluorescens* Pf0-1 and *Pseudomonas syringae*, and lower homology with other pseudomonads including *P. aeruginosa* and *Pseudomonas putida* (58–62 %). It also shows limited but significant homology (37 %) with the enterobacterial FliS proteins. Similarly to the enterobacterial FliS proteins, the F113 FliS has a putative amphipathic alpha-helix in the carboxy-terminus. The second ORF (*fliT*) is similar in size to ORFs with the same location in other genomes, although homology between them is very low (Table 2.2). This ORF has been assigned to the *orf96* gene in *P. aeruginosa* PAK (Arora *et al.*, 1998), the *fleP* gene in *P. aeruginosa* PAO1 strain (Dasgupta *et al.*, 2003), and *fliT* in Salmonella (Bennett *et al.*, 2001).

Overall, the genetic organization of this region is identical to that of *P. fluorescens* Pf0-1, *P. aeruginosa* PAO1, *P. putida* KT2440 and several pathovars of

P. syringae (Nelson *et al.*, 2002). However, it differs from the gene order in *P. aeruginosa* strains containing type a flagellin such as PAK (GenBank accession no. L81176) and DG1 (GenBank accession no. L43064), which contain an extra copy of a gene similar to *fliS*, called *fliS´*, located downstream of *fliS*.

Mutants affected in each of these genes were generated by insertional mutagenesis by cloning an internal fragment of the gene in plasmid pVIK112 (Kalogeraki & Winans, 1997) and homologous recombination in the wild-type strain; this resulted in non-polar mutations, as determined by genetic complementation (see below). Despite several efforts, we have been unable to obtain mutants in the *fliD* gene, since besides the insertion, a wild-type copy of the gene was generated.

All the mutants obtained were grown in SA and LB liquid media and showed no differences in growth parameters from the wild-type strain.

The 6.5 kb region sequence has been deposited in GenBank with the accession number AF399739.

Morphological analysis of mutants

Flagellar and cell morphology of *P. fluorescens* F113 and the mutants in the flagellar synthesis region were studied by transmission electron microscopy (Fig. 2.2). The F113 wild-type strain possesses one or two polar flagella of mean length 2.4 μm (Sánchez-Contreras *et al.*, 2002). The mutations located within the *fliC* and the *fleQ* genes resulted in completely aflagellate bacterial cells. The presence of flagella was restored by the introduction of plasmids containing the wild-type genes under the control of the *nptII* promoter. The mutant affected in the *fliS* gene had a single thin and very short flagellum of about 0.8 μm in length. Introduction of the wild-type gene into this mutant resulted in normal flagellate

cells. Conversely, the mutation in the *flaG* gene resulted in bacterial cells with one or two very long (more than 5 μm) flagellar filaments. Finally, the mutation affecting the *fliT* gene had no visible effect, and bacterial cells had flagella with identical morphology to those of the wild-type strain.

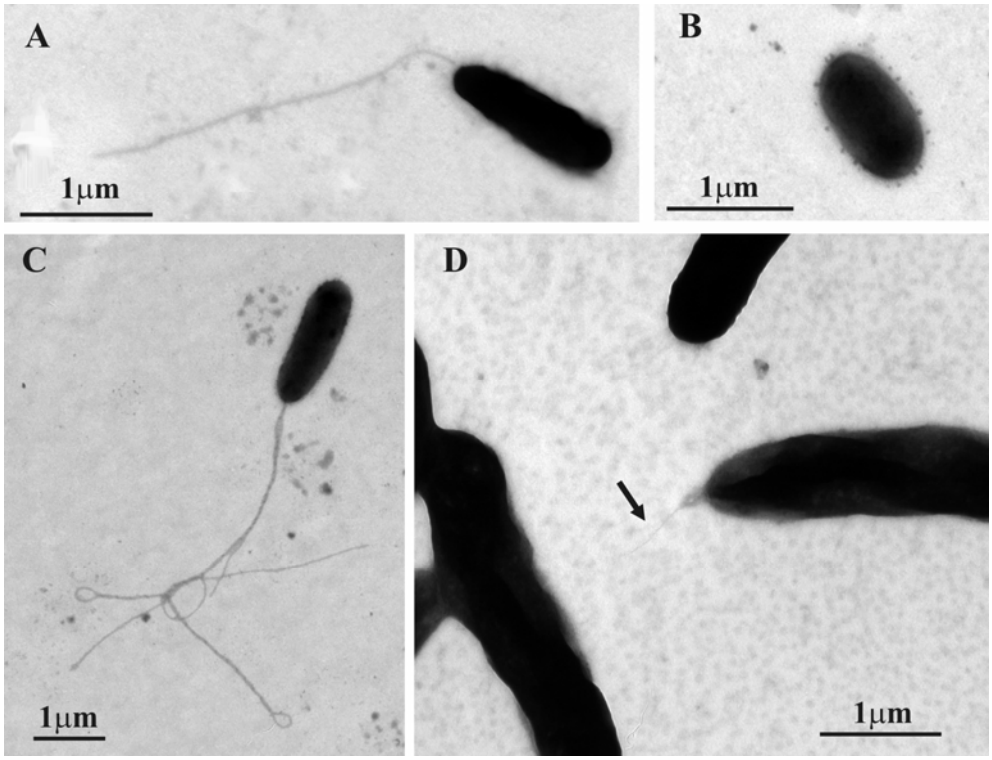

Figure 2.2. Electron microscopy images of F113 wild-type and flagellar filament mutants. (A) F113 wild-type. (B) F113-fliC. (C) F113-flaG. (D) F113-fliS. Arrow points to a thin, short flagellar filament produced by the *fliS* mutant. Flagella of the *fliT* mutant are identical to wild-type. The *fleQ* mutant does not produce flagella.

Analysis of the motility phenotype

In order to study the motility characteristics of the mutants, we analysed their ability to swim (Fig. 2.3). After 18 h the wild-type strain produces a 6–7 mm diameter swimming halo. Mutations located within the *fliC*, *fleQ* and *fliS* genes resulted in non-motile mutants as they did not produce swimming haloes. Complementation of these mutants with the wild-type genes restored motility to 100 %, 85% and 85% of the wild-type, respectively.

The mutant affected in the *flaG* gene, which had longer flagellar filaments than the F113 wild-type, produced haloes similar to those of the wild-type strain. However, when swimming experiments were performed in richer medium such as LB or iron-supplemented SA, this mutant produced swimming haloes with a diameter 50–100% wider than those from the wild-type strain.

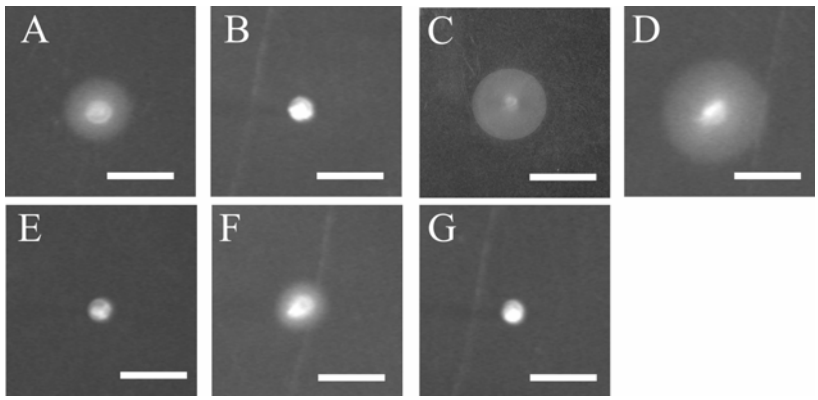

Figure 2.3. Swimming haloes produced on 0.3 % agar plates by: (A) F113 on SA. (B) F113-fliC on SA. (C) F113-flaG on SA. (D) F113-flaG on LB. (E) F113-fliS on SA. (F) F113-fliT on SA. (G) F113-fleQ on SA. The bar represents the diameter of the wild-type strain halo in the same swimming conditions.

The *fliT* mutant, whose filament morphology was similar to the wild-type strain, produced swimming haloes 50% smaller than the wild-type. Complementation of the *fliT* mutant restored motility to 83% of the wild-type. It is important to note that the *fliT* mutant haloes, although smaller, presented clear concentric circles inside them, typical of the chemotactic swimming movement.

Flagellin synthesis and export

Total bacterial proteins and exported proteins were analysed by Western blotting with an anti-flagellin antiserum (de Weger *et al.*, 1987) (Fig. 2.4). The F113 wild-type strain gave a band corresponding to the FliC flagellin, both in the total protein and in the exported protein preparations. The same results were obtained with the *fliT* mutant.

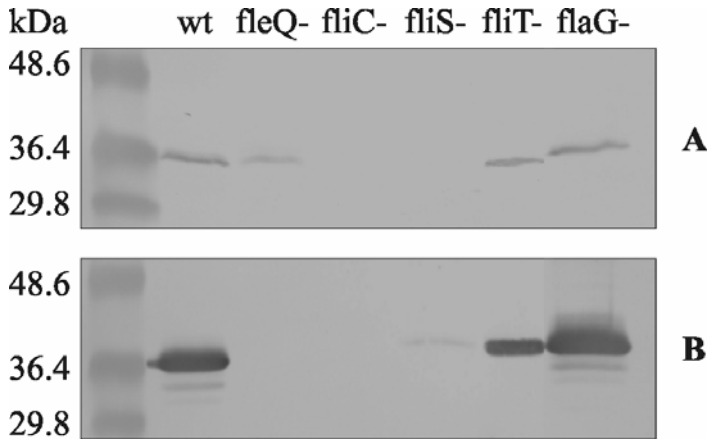

Figure 2.4. Western blot analysis of total proteins (A) and external proteins (B) from flagellar filament mutants and wild-type F113, reacted with an anti-flagellin antiserum. The observed band is approximately 35 KDa and corresponds to FliC.

As expected, the *fliC* mutant did not produce flagellin and no band was detected in either of the protein preparations. The mutation affecting the *fleQ* gene produced very low levels of flagellin in the experiment done with the total proteins; no band appeared in the case of exported proteins.

In the *flaG* mutant, the Western blot revealed a normal level of flagellin in the total protein extract and a higher level of exported flagellin, in accordance with its longer flagellar filament morphology.

In the case of the *fliS* mutant, we did not observe a band in the total protein Western blot analysis, although a very faint band appeared in the exported proteins extract. In order to understand the results obtained with the *fliS* mutant Western blot experiment, a dot-blot analysis with a whole-cell lysate (soluble and non-soluble proteins) was performed. The antiflagellin antiserum gave a very strong reaction with this bacterial lysate (data not shown). These results indicate that in the *fliS* mutant, most of the FliC protein is probably being accumulated in inclusion bodies formed inside the cell cytoplasm.

Colonization analysis of flagellar mutants

Colonization experiments with each of the mutants inoculated individually showed that all the mutants constructed in this work were able to colonize the alfalfa rhizosphere (data not shown).

In order to study the importance of bacterial motility in the rhizosphere colonization process, we analysed the competitiveness between the flagellar filament mutants and the wild-type F113 strain. A wild-type F113 strain tagged in a neutral part of the genome with the same integration plasmid (pVIK112) that had been used to generate the mutants was used as the competitor strain.

As shown in Fig. 2.5, all the non-motile mutants, *fliC*, *fliS* and *fleQ*, were very poor competitors and were displaced by the wild-type strain from the last centimetre of the root. In a similar way, the *fliT* mutant that showed reduced motility was displaced by the wild-type F113 strain. No significant differences were observed between the competitive colonization ability of non-motile and reduced-motility mutants. The control strain and the *flaG* mutant competed at the same level as the wild-type strain F113 under our laboratory gnotobiotic competitive assay conditions.

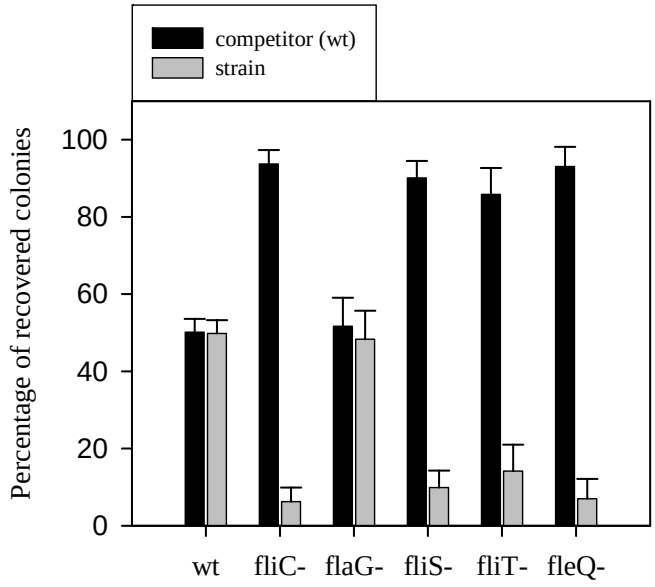

Figure 2.5. Competitive root tip colonization analysis of flagellar filament mutants and wild-type F113 competitor strain. Black bars represent percentage of competitor colonies and grey bars represent percentage of each tested strain recovered from the last centimetre of the main root after competitive colonization assays. Means and standard deviations from three independent assays, performed in triplicate, are shown.

DISCUSSION

The study of bacterial motility and its influence on root colonization and competition in the rhizosphere may eventually result in improved efficacy of biotechnological applications. It is already known that bacterial motility is important in the colonization of the rhizosphere, since different non-motile mutants from *P. fluorescens* strain WCS374 were severely impaired in colonization (Dekkers *et al.*, 1998a). In fact, the non-motile mutants belong to the most defective competitive class of colonization mutants (Chin-A-Woeng *et al.*, 2000; Dekkers *et al.*, 1998b).

In this work we have characterized a genetic region of *P. fluorescens* F113 implicated in the synthesis of the flagellar filament and we have shown that the genetic organization is similar to other pseudomonads but differs from the *P. aeruginosa* PAK and DG1 strains because they have two copies of the *fliS* gene.

The first gene in the region, *fliC,* encodes a type b flagellin, the main structural protein of the flagellar filament. As expected, the mutation of this gene yields completely non-motile and aflagellate bacteria. In the mutation affecting the *fleQ* gene we have found the same morphological and non-motile phenotype. These results are in agreement with the already described function for the FleQ protein, which is the major flagellar regulator in *P. aeruginosa* (Dasgupta *et al.*, 2002; Jyot *et al.*, 2002). In *P. fluorescens* Pf0-1, a *fleQ* homologue gene called *adnA* encodes a transcriptional factor that affects persistence and spread, also being required for bacterial adhesion and motility (Casaz *et al.*, 2001; Marshall *et al.*, 2001). From the F113 *fleQ* mutant phenotype (Figs 2.2 and 2.3) and the results from the Western blot analysis (Fig. 2.4), it can be concluded that in *P. fluorescens* F113 the *fleQ* gene is necessary for the production and secretion of the flagellin FliC.

Another mutation causing non-motile cells affects the *fliS* gene. In pseudomonads the role of the FliS protein remains unknown. Its distant homologue in enterobacteria has been described as a substrate-specific cytosolic chaperone that facilitates FliC secretion and contributes to the stabilization of the flagellin subunits during polymerization (Auvray *et al.*, 2001; Ozin *et al.*, 2003). The F113 *fliS* mutant has a very short and thin flagellum (Fig. 2.2), probably because FliC is not well stabilized and is undergoing wrong polymerization and limited secretion, thus impairing the formation of a normal flagellar filament. To our knowledge, this is the first description of such a phenotype. The results from the *fliS* mutant Western blot analysis confirm the FliS putative function as a FliC chaperone. These results indicate that, instead of being secreted, the FliC flagellin is accumulated in the cytoplasm and as these proteins cannot be extracted together with the total soluble bacterial proteins, the flagellin might be accumulated inside the cytoplasm inclusion bodies. Moreover, the structural analysis of the *P. fluorescens* F113 FliS protein shows that it is homologous to other FliS proteins, being a small peptide, with an acidic isoelectric point (5.18) and having an amphipathic alpha-helix in the C-terminal domain, typical characteristics for most cytoplasmic chaperones (Fraser *et al.*, 1999; Wattiau *et al.*, 1996).

Downstream of *fliS*, the pseudomonads contain a small ORF showing very low homology with the *fliT* genes of enterobacteria (Table 2.2). In these bacteria, the FliT protein has been described as the FliD substrate-specific chaperone (Fraser *et al.*, 1999), although motility studies done with the *Salmonella typhimurium fliT* mutant concluded that there were no differences in the swimming ability compared with the wild-type strain (Bennett *et al.*, 2001). In *P. fluorescens* F113, the morphological phenotype of the *fliT* mutant was identical to the wild-type strain and the Western blot analysis revealed that the FliC protein is exported to form the flagellar filament. Therefore the putative protein FliT cannot be acting as a FliD cytosolic chaperone. Furthermore, the structural characteristics of the FliT protein are different to those described for cytoplasmic chaperones. In *P. aeruginosa* PAK,

an ORF similar in size and gene location to *fliT* has been designed as *fleP*, encoding a hypothetical protein FleP. The swimming haloes produced by a mutation affecting the *fleP* gene are much smaller than those produced by the *P. aeruginosa* wild-type strain (Dasgupta *et al.*, 2003). This *fleP* mutant motility phenotype is similar to the *P. fluorescens* F113 *fliT* mutant phenotype, which produced swimming haloes 50% smaller than the wild-type strain. However, electron microscopy studies of the mutant affected in the *fleP* gene in *P. aeruginosa* PAK revealed that its flagella were mostly detached from the cells and the length of polar type IV pili was significantly longer than those from the wild-type strain (Dasgupta *et al.*, 2003). Based on these results, the authors concluded that *fleP* represents a novel flagellar gene specific for *Pseudomonas*, responsible for maintaining the length of type IV pili and stable flagellar attachment to the bacterial pole. These results do not correlate with the normal flagellar morphology observed for our *P. fluorescens* F113 *fliT* mutant (Fig. 2.2). Such differences, together with the lack of homology between them (Table 2.2), indicate that the *fleP* gene in *P. aeruginosa* and the *fliT* gene in *P. fluorescens* are different and possess different functions.

A mutant affected in the *flaG* gene had longer flagella than the wild-type F113 (Fig. 2.2) and flagellin was clearly exported in a higher quantity than in the wild-type strain as observed by Western blot analysis (Fig. 2.4). These characteristics did not result in higher motility properties in an iron-limited minimal medium, and the *flaG* mutant produced swimming haloes with a diameter similar to that of the wild-type strain (Fig. 2.3). However, in rich medium or in the absence of iron limitation, this mutant showed a substantially higher motility (Fig. 2.3). In *V. anguillarum*, an ORF 3 showing a 57% identity to FlaG from *Vibrio parahaemolyticus* and 34% identity to FlaG from *P. fluorescens* F113 has been described, and a mutant affected in this gene had elongated flagella, the same morphological phenotype as in the *P. fluorescens* F113 *flaG* mutant, although it showed an 11% decrease compared with the wild-type motility (McGee *et al.*,

1996). These results indicate that in addition to filament length, FlaG could influence swimming speed, at least under certain conditions.

Root colonization is a complex and crucial process for the use of micro-organisms for agricultural and environmental biotechnology applications, since an improvement in colonization could result in an improvement in the efficacy of these applications, as has been shown for biocontrol (Chin-A-Woeng *et al.*, 2000). Most of the already described non-motile mutants are severely affected in root colonization, especially at sites most distant from the inoculation site (de Weger *et al.*, 1987). We have also observed the predominance of flagellar variants with enhanced surface motility, in the distal parts of the rhizosphere that are not easily reached by the wild-type strain (Sánchez-Contreras *et al.*, 2002).

All the non-motile *P. fluorescens* F113 mutants studied in this work (*fliC*, *fleQ*, *fliS*) although able to colonize when inoculated independently, were very poor competitors, compared with the wild-type F113. These results are in agreement with previously published work that shows that aflagellate (de Weger *et al.*, 1987) or non-chemotactic mutants (de Weert *et al.*, 2002) are very poor competitors. We have isolated and tested a mutant affected in the *fliT* gene that, although still motile and chemotactic, showed a reduced motility phenotype. This mutant was as poor a competitor as aflagellate mutants, showing that not only flagella and chemotactic motility (de Weert *et al.*, 2002), but also a wild-type level of motility are necessary for competitive rhizosphere colonization. Furthermore, although no differences in motility or colonization were observed for a *flaG* mutant under the standard conditions used, the fact that this mutant showed higher motility under certain conditions and the preferred location of hypermotile variants in distal parts of the root (Sánchez-Contreras *et al.*, 2002) suggest the possibility of improving competitive root colonization by manipulating the motility processes.

ACKNOWLEDGEMENTS

We are grateful to Ine Mulders and Ben Lugtenberg for the anti-flagellin antiserum. This work was supported by grants from the European Union (GM-RHIZOREMEDIATION QLK3-CT-2001-00101), Comunidad Autónoma de Madrid (07 M-0062-2000) and Spanish Ministry of Science and Technology BIO 2003-03412. M. M. is the recipient of a Ramón y Cajal contract from the Spanish Ministry of Science and Technology.

REFERENCES

Arora, S. K., Ritchings, B. W., Almira, E. C., Lory, S. & Ramphal, R. (1998). The *Pseudomonas aeruginosa* flagellar cap protein, FliD, is responsible for mucin adhesion. *Infect Immun* **66**, 1000-1007.

Arora, S. K., Dasgupta, N., Lory, S. & Ramphal, R. (2000). Identification of two distinct types of flagellar cap proteins, FliD, in *Pseudomonas aeruginosa*. *Infect Immun* **68**, 1474-1479.

Auvray, F., Thomas, J., Fraser, G. M. & Hughes, C. (2001). Flagellin polymerisation control by a cytosolic export chaperone. *J Mol Biol* **308**, 221-229.

Bennett, J. C. Q., Thomas, J., Fraser, G. M. & Hughes, C. (2001). Substrate complexes and domain organization of the *Salmonella flagellar* export chaperones FlgN and FliT. *Mol Microbiol* **39**, 781-791.

Bertani, G. (1951). Studies on Lysogenesis. 1. The Mode of Phage Liberation by Lysogenic *Escherichia coli*. *J Bacteriol* **62**, 293-300.

Bloemberg, G. V., Wijfjes, A. H. M., Lamers, G. E. M., Stuurman, N. & Lugtenberg, B. J. J. (2000). Simultaneous imaging of *Pseudomonas fluorescens* WCS365 populations expressing three different autofluorescent proteins in the rhizosphere: New perspectives for studying microbial communities. *Mol Plant-Microbe Interact* **13**, 1170-1176.

Brazil, G. M., Kenefick, L., Callanan, M., Haro, A., de Lorenzo, V., Dowling, D. N. & O´Gara, F. (1995). Construction of a rhizosphere pseudomonad with potential to degrade polychlorinated biphenyls and detection of *bph* gene expression in the rhizosphere. *Appl Environ Microbiol* **61**, 1946-1952.

Casaz, P., Happel, A., Keithan, J., Read, D. L., Strain, S. R. & Levy, S. B. (2001). The *Pseudomonas fluorescens* transcription activator AdnA is required for adhesion and motility. *Microbiol* **147**, 355-361.

Chin-A-Woeng, T. F. C., Bloemberg, G. V., Mulders, I. H. M., Dekkers, L. C. & Lugtenberg, B. J. J. (2000). Root colonization by phenazine-1-carboxamide-producing bacterium *Pseudomonas chlororaphis* PCL1391 is essential for biocontrol of tomato foot and root rot. *Mol Plant-Microbe Interact* **13**, 1340-1345.

Dasgupta, N., Ferrell, E. P., Kanack, K. J., West, S. E. H. & Ramphal, R. (2002). *fleQ*, the gene encoding the major flagellar regulator of *Pseudomonas aeruginosa*, is σ^{70} dependent and is downregulated by Vfr, a homolog of *Escherichia coli* cyclic AMP receptor protein. *J Bacteriol* **184**, 5240-5250.

Dasgupta, N., Wolfgang, M. C., Goodman, A. L., Arora, S. K., Jyot, J., Lory, S. & Ramphal, R. (2003). A four-tiered transcriptional regulatory circuit controls flagellar biogenesis in *Pseudomonas aeruginosa*. *Mol Microbiol* **50**, 809-824.

de Weert, S., Vermeiren, H., Mulders, I. H. M., Kuiper, I., Hendrickx, N., Bloemberg, G. V., Vanderleyden, J., de Mot, R. & Lugtenberg, B. J. J. (2002). Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by *Pseudomonas fluorescens*. *Mol Plant-Microbe Interact* **15**, 1173-1180.

de Weger, L. A., van der vlugt, C. I. M., Wijfjes, A. H. M., Bakker, P. A. H. M., Schippers, B. & Lugtenberg, B. (1987). Flagella of a plant-growth-stimulating *Pseudomonas fluorescens* strain are required for colonization of potato roots. *J Bacteriol* **169**, 2769-2773.

Dekkers, L. C., Phoelich, C. C., van der Fits, L. & Lugtenberg, B. J. J. (1998a). A site-specific recombinase is required for competitive root colonization by *Pseudomonas fluorescens* WCS365. *Proc Natl Acad Sci U S A* **95**, 7051-7056.

Dekkers, L. C., van der Bij, A. J., Mulders, I. H. M., Phoelich, C. C., Wentwoord, R. A. R., Glandorf, D. C. M., Wijffelman, C. A. & Lugtenberg, B. J. J. (1998b). Role of the O-antigen of lipopolysaccharide, and possible roles of growth rate and of NADH : ubiquinone oxidoreductase (Nuo) in competitive tomato root-tip colonization by *Pseudomonas fluorescens* WCS365. *Mol Plant-Microbe Interact* **11**, 763-771.

Dowling, D. N. & O´Gara, F. (1994). Metabolites of *Pseudomonas* Involved in the biocontrol of plant-disease. *Trends Biotechnol* **12**, 133-141.

Fahraeus, G. (1957). The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. *J Gen Microbiol* **16**, 374-381.

Figurski, D. H. & Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci U S A* **76**, 1648-1652.

Fraser, G. M., Bennett, J. C. Q. & Hughes, C. (1999). Substrate-specific binding of hook-associated proteins by FlgN and FliT, putative chaperones for flagellum assembly. *Mol Microbiol* **32**, 569-580.

Höflich, G., Wiehe, W. & Hechtbuchholz, C. (1995). Rhizosphere colonization of different crops with growth-promoting *Pseudomonas* and *Rhizobium* bacteria. *Microbiol Res* **150**, 139-147.

Jyot, J., Dasgupta, N. & Ramphal, R. (2002). *fleQ*, the major flagellar gene regulator in *Pseudomonas aeruginosa*, binds to enhancer sites located either upstream or atypically downstream of the RpoN binding site. *J Bacteriol* **184**, 5251-5260.

Kalogeraki, V. S. & Winans, S. C. (1997). Suicide plasmids containing promoterless reporter genes can simultaneously disrupt and create fusions to target genes of diverse bacteria. *Gene* **188**, 69-75.

Karlson, U., Dowling, D., O´Gara, F., Rivilla, R., Bittens, M., Francesconi, S., Pritchard, H. & Pederson, H. C. (1998). Development of self-contained plant/GMM system for soil bioremediation. In *Past, Present and Future Risk Assessment when using GMOs*, pp. 23-31. Edited by G. E. d. Vries. Overschild, NL: Probio Partners.

Labes, M., Puhler, A. & Simon, R. (1990). A new family of RSF1010-derived expression and *lac*-fusion broad-host-range vectors for Gram-negative bacteria. *Gene* **89**, 37-46.

Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of head of bacteriophage T4. *Nature* **227**, 680-685.

Lugtenberg, B. J. J. & Dekkers, L. C. (1999). What makes *Pseudomonas* bacteria rhizosphere competent?. *Environ Microbiol* **1**, 9-13.

Lugtenberg, B. J. J., Dekkers, L. & Bloemberg, G. V. (2001). Molecular determinants of rhizosphere colonization by *Pseudomonas*. *Annu Rev Phytopathol* **39**, 461-490.

Marshall, B., Robleto, E. A., Wetzler, R., Kulle, P., Casaz, P. & Levy, S. B. (2001). The *adnA* transcriptional factor affects persistence and spread of *Pseudomonas fluorescens* under natural field conditions. *Appl Environ Microbiol* **67**, 852-857.

McGee, K., Horstedt, P. & Milton, D. L. (1996). Identification and characterization of additional flagellin genes from *Vibrio anguillarum*. *J Bacteriol* **178**, 5188-5198.

Naseby, D. C. & Lynch, J. M. (1998). Impact of wild-type and genetically modified *Pseudomonas fluorescens* on soil enzyme activities and microbial population structure in the rhizosphere of pea. *Mol Ecol* **7**, 617-625.

Nelson, K. E., Weinel, C., Paulsen, I. T. & other authors (2002). Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ Microbiol* **4**, 799-808.

Ozin, A. J., Claret, L., Auvray, F. & Hughes, C. (2003). The FliS chaperone selectively binds the disordered flagellin C-terminal D0 domain central to polymerisation. *FEMS Microbiol Lett* **219**, 219-224.

Rainey, P. B. (1999). Adaptation of *Pseudomonas fluorescens* to the plant rhizosphere. *Environ Microbiol* **1**, 243-257.

Ramos, J. L., Duque, E. & Ramos-González, M. I. (1991). Survival in soils of an herbicide-resistant *Pseudomonas putida* strain bearing a recombinant Tol plasmid. *Appl Environ Microbiol* **57**, 260-266.

Robleto, E. A., López-Hernández, I., Silby, M. W. & Levy, S. B. (2003). Genetic analysis of the AdnA regulon in *Pseudomonas fluorescens*: nonessential role of flagella in adhesion to sand and biofilm formation. *J Bacteriol* **185**, 453-460.

Sambrook, J., Fritsch, E. F. & Maniatics, T. (1989). Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sánchez-Contreras, M., Martín, M., Villacieros, M., O'Gara, F., Bonilla, I. & Rivilla, R. (2002). Phenotypic selection and phase variation occur during alfalfa root colonization by *Pseudomonas fluorescens* F113. *J Bacteriol* **184**, 1587-1596.

Scher, F. M. & Baker, R. (1982). Effects of *Pseudomonas putida* and a synthetic iron chelator on induction of soil suppressiveness to *Fusarium wilt* pathogens. *Phytopathology* **72**, 1567-1573.

Shanahan, P., O´Sullivan, D. J., Simpson, P., Glennon, J. D. & O´Gara, F. (1992). Isolation of 2,4-diacetylphloroglucinol from a fluorescent pseudomonad and investigation of physiological parameters influencing its production. *Appl Environ Microbiol* **58**, 353-358.

Simons, M., van der Bij, A. J., Brand, I., de Weger, L. A., Wijffelman, C. A. & Lugtenberg, B. J. J. (1996). Gnotobiotic system for studying rhizosphere colonization by plant growth-promoting *Pseudomonas* bacteria. *Mol Plant-Microbe Interact* **9**, 600-607.

Stover, C. K., Pham, X. Q., Erwin, A. L. & other authors (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* **406**, 959-964.

Villacieros, M., Power, B., Sánchez-Contreras, M. & other authors (2003). Colonization behaviour of *Pseudomonas fluorescens* and *Sinorhizobium meliloti* in the alfalfa (*Medicago sativa*) rhizosphere. *Plant Soil* **251**, 47-54.

Walsh, U. F., Morrissey, J. P. & O'Gara, F. (2001). Pseudomonas for biocontrol of phytopathogens: from functional genomics to commercial exploitation. *Curr Opin Biotechnol* **12**, 289-295.

Wattiau, P., Woestyn, S. & Cornelis, G. R. (1996). Customized secretion chaperones in pathogenic bacteria. *Mol Microbiol* **20**, 255-262.

Yee, D. C., Maynard, J. A. & Wood, T. K. (1998). Rhizoremediation of trichloroethylene by a recombinant, root-colonizing *Pseudomonas fluorescens* strain expressing toluene ortho-monoooxygenase constitutively. *Appl Environ Microbiol* **64**, 112-118.

CAPÍTULO 3

Two site-specific recombinases are implicated in phenotypic variation and competitive rhizosphere colonization in *Pseudomonas fluorescens*

ABSTRACT

The biocontrol agent *Pseudomonas fluorescens* F113 undergoes phenotypic variation during rhizosphere colonization, and this variation has been related to the activity of a site-specific recombinase encoded by the *sss* gene. Here, it is shown that a second recombinase encoded by the *xerD* gene is also implicated in phenotypic variation. A putative *xerD* gene from this strain was cloned, and sequence analysis confirmed that it encoded a site-specific recombinase of the λ integrase family. Mutants affected in the *sss* or *xerD* genes produced a very low quantity of phenotypic variants compared to the wild-type strain, both under prolonged cultivation in the laboratory and after rhizosphere colonization and they were severely impaired in competitive root colonization. Overexpression of the genes encoding either recombinase resulted in a substantial increment in the production of phenotypic variants under both culture and rhizosphere colonization conditions, implying that both site-specific recombinases are involved in phenotypic variation. Overexpression of the *sss* gene suppressed the phenotype of a *xerD* mutant, but overexpression of the *xerD* gene had no effect on the phenotype of an *sss* mutant. Genetic analysis of the phenotypic variants obtained after overexpression of the genes encoding both the recombinases showed that they carried mutations in the *gacA/S* genes, which are necessary to produce a variety of secondary metabolites. These results indicate that the Gac system is affected by the activity of the site-specific recombinases. Transcriptional fusions of the *sss* and *xerD* genes with a promoterless *lacZ* gene showed that both genes have a similar expression pattern, with maximal expression during stationary phase. Although the expression of both genes was independent of diffusible compounds present in root exudates, it was induced by the plant, since bacteria attached to the root showed enhanced expression.

INTRODUCTION

Site-specific recombinases catalyse genetic rearrangements related to multiple processes such as plasmid (Colloms *et al.*, 1990) and chromosome segregation (Blakely *et al.*, 1991), phage integration (reviewed by Groth and Calos (2004)) and phase variation (Dove & Dorman, 1994; Tominaga *et al.*, 1991). One of the best characterized is the *Escherichia coli* recombinase XerC, which forms an heterotetramer with the recombinase XerD (Ferreira *et al.*, 2003), and participates in chromosome segregation after replication (Blakely *et al.*, 1991; Blakely *et al.*, 1993), and in the segregation of different plasmids such as ColE1 (Blakely *et al.*, 1993; Colloms *et al.*, 1990) and pSC101 (Cornet *et al.*, 1994), resolving plasmid dimers.

The pseudomonads possess orthologues of genes encoding these site-specific recombinases. A gene from *Pseudomonas aeruginosa* affecting pyoverdine production, and named *sss*, was shown to be homologous to the *E. coli xerC* gene (Hofte *et al.*, 1994). The *sss* gene has also been cloned from different strains of *Pseudomonas fluorescens* (Dekkers *et al.*, 1998; Sánchez-Contreras *et al.*, 2002). An *sss* mutant of *P. fluorescens* WCS365 was affected in competitive rhizosphere colonization, and was displaced by the wild-type from the root tip of a variety of plants (Dekkers *et al.*, 1998). It has also been shown that introduction of extra copies of the *sss* gene can improve rhizosphere colonization (Dekkers *et al.*, 2000) and biocontrol abilities of different pseudomonads (Chin-A-Woeng *et al.*, 2000). A second recombinase that might function in conjunction with Sss is also present in pseudomonads, since the sequences of the *P. aeruginosa* PAO1 and *P. fluorescens* Pf0-1 genomes have shown the presence of genes homologous to the *E. coli xerD* genes. To date, these *xerD* homologues have not been cloned or analysed.

Phenotypic (phase) variation in pseudomonads occurs during rhizosphere colonization, and phenotypic variants showing a translucent and diffuse colony morphology have been isolated in laboratory cultures (van den Broek *et al.*, 2003),

and after rhizosphere passage (Achouak *et al.*, 2004; Sánchez-Contreras *et al.*, 2002). In *P. fluorescens* F113, the phenotypic variants isolated from the alfalfa rhizosphere showed enhanced motility, and their numbers were significantly reduced by a mutation in the *sss* gene (Sánchez-Contreras *et al.*, 2002). Most of the few variants observed among the *sss* mutants also carried a secondary mutation affecting the Gac system (Sánchez-Contreras *et al.*, 2002). The Gac system, forming a two-component regulatory system encoded by the *gacA* and the *gacS* (*lemA*) genes, regulates the production of secondary metabolites such as exoprotease and cyanide (Blumer *et al.*, 1999). This system has also been implicated in phase variation in *Pseudomonas* sp. PCL1171 (van den Broek *et al.*, 2003), since mutants in the *gacS* gene were locked in phase II, which is morphologically equivalent to the phenotypic variants produced by *P. fluorescens* F113 after rhizosphere colonization.

Due to the importance of phenotypic variation for rhizosphere colonization, and to improve biotechnological applications of these bacteria in biocontrol and rhizoremediation, the aim of this work was to characterize the *xerD* gene of *P. fluorescens* F113, and to determine its possible role in phenotypic variation and rhizosphere colonization.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Strains and plasmids used in this study are shown in Table 3.1. All the *P. fluorescens* strains are derivatives of the biocontrol strain F113, which was isolated from the sugarbeet rhizosphere (Shanahan *et al.*, 1992). The F113 genebank used was constructed with partially EcoRI-digested genomic DNA cloned into the pLAFR3 cosmid. The *sss* and *xerD* mutants were generated by gene disruption using the suicide vector pK18*mobsac* (Schafer *et al.*, 1994). Mutants were checked by Southern blotting. Overexpression of genes was achieved by cloning them under the

control of the strong *nptII* promoter present in the pFAJ1709 plasmid (Dombrecht *et al.*, 2001). All plasmids were mobilized to *P. fluorescens* by triparental matings, using pRK2013 as the helper plasmid (Figurski & Helinski, 1979).

Table 3.1. Strains and plasmids used in this study

Strain /plasmid	Relevant characteristics	Source or reference
Strains		
DH5α	General purpose *Escherichia coli* strain	(Boyer & Roulland-Dussoix, 1969)
F113rif	*Pseudomonas fluorescens* wild-type strain, Rif^r	(Shanahan *et al.*, 1992)
F113-sss2	F113rif *sss* mutant, Rif^r, Km^r	(Sánchez-Contreras *et al.*, 2002)
F113-xerD	F113rif *xerD* mutant, Rif^r, Km^r	This work
F113 *sss*::*lac*	F113rif *sss::lacZ*, Rif^r, Km^r	This work
F113 *xerD*::*lac*	F113rif *xerD::lacZ*, Rif^r, Km^r	This work
Plasmids		
pK18*mobsacB*	pUC18 derivative *lacZ mob* site *sacB*, Km^r	(Schafer *et al.*, 1994)
pBG1261	pK18*mobsac* with an internal fragment of the *xerD* gene, used to generate F113-xerD	This work
pFAJ1709	Expression vector, Tet^r	(Dombrecht *et al.*, 2001)
pBG1457	pFAJ1709 derivative carrying *sss* under the control of the *nptII* promoter, used to overexpress *sss*	This work
pBG1442	pFAJ1709 derivative carrying *xerD* under the control of the *nptII* promoter, used to overexpress *xerD*	This work
pVIK112	*lacZ* reporter suicide vector, Km^r	(Kalogeraki & Winans, 1997)
pBG1319	pVIK112 *sss::lacZ* transcriptional fusion	This work
pBG1306	pVIK112 *xerD::lacZ* transcriptional fusion	This work
pRK2013	Helper plasmid used in triparental matings, Km^r	(Figurski & Helinski, 1979)
pME3066	A 1.65 Kb BamHI-BglII fragment containing the *gacA* gene from *Pseudomonas fluorescens* CHAO in pVK100, Tet^r	(Laville *et al.*, 1992)
pEMH97	A 9.7-Kb HindIII fragment containing the *gacS* gene from *Pseudomonas syringae* in pLAFR3, Tet^r	(Hrabak & Willis, 1992)

All *P. fluorescens* strains were grown in SA medium (Scher & Baker, 1982) overnight at 28 ºC. *E. coli* strains were grown overnight in Luria–Bertani (LB) medium (Bertani, 1951) at 37 ºC. For the prolonged laboratory growth, bacteria were grown for 1 week in SA liquid medium with shaking, and the cultures were plated on solidified SA medium. When required, the following antibiotics were used at the indicated concentrations: rifampicin 100 μg/ml, tetracycline 10 μg/ml (for *E. coli*) or 70 μg/ml (for *P. fluorescens*), and kanamycin 25 μg/ml (for *E. coli*) or 50 μg/ml (for *P. fluorescens*). Exoprotease production was observed on skim-milk plates, as described by Sacherer *et al*. (1994). The same plates were used to assess pyoverdine production by inspection under UV light.

DNA manipulations and sequence analysis

Standard methods were used for DNA extraction and gene cloning (Sambrook *et al.*, 1989). Southern blotting and colony hybridization were performed with a non-radioactive detection kit, and a chemiluminescence method was used to detect hybridization signals according to the instructions of the manufacturer (Boehringer Mannheim). PCR reactions were performed using the *Tth* enzyme (Biotools) under standard conditions. Primer sequences are available on request. DNA was custom sequenced by Centro Nacional de Investigaciones Oncológicas (CNIO; Madrid, Spain) and Sistemas Genómicos (Valencia, Spain). Sequence analysis was performed with software from the Genetics Computer Group (Madison, WI, USA) and the BLAST programs.

Protein sequences were aligned by using the multiple-sequence-alignment tool CLUSTAL W (Thompson *et al.*, 1994). The aligned sequences were studied by using genetic distance and bootstrap NJ tree for phylogenetic inference (1000 iterations). Ambiguous characteristics (where a deletion, insertion or unidentified state was recorded for any strain) were removed from the alignment data.

Rhizosphere colonization experiments

Alfalfa seeds were sterilized in 70% ethanol for 2 min, diluted bleach (1:5) for 15 min, and then rinsed thoroughly with sterile distilled water. Seed vernalization was performed at 4 ºC overnight, and germination was for 1 day at 28 ºC. Germinated alfalfa seeds were sown in Leonard jar gnotobiotic systems (Villacieros *et al.*, 2003) using perlite as the solid substrate, and 8 mM KNO_3-supplemented Fahraeus (FP) medium (Fahraeus, 1957) as the mineral solution. After 2 days, alfalfa seeds were inoculated with ~10^8 cells of the appropriate strains. In competition experiments, strains were inoculated at a 1:1 ratio. Plants were maintained in controlled conditions (16 h light at 25 ºC, and 8 h dark at 18 ºC) for 3 weeks. Bacteria were recovered from the rhizosphere by vortexing the root tips (last centimetre of the main root) for 2 min in a tube containing 5 ml 0.9% NaCl, and plating the appropriate dilutions on SA agar containing antibiotics. Each experiment was performed in triplicate, and each replicate contained at least 20 plants. Statistical analysis was done with Sigma Plot 4 software (Windows).

Gene expression analysis

Transcriptional *lacZ* fusions of the *sss* and *xerD* genes were constructed by directionally cloning an internal fragment of the gene into the pVIK112 suicide vector (Kalogeraki & Winans, 1997). The construct was integrated into the F113 genome by triparental mating, and single homologous recombinants were checked by PCR and Southern blotting.

Cells harbouring these constructs were grown in LB medium, and samples were taken at intervals. β-Galactosidase activity was determined according to Miller (1972).

Aqueous and methanolic root exudates were prepared by extracting roots from 100 5-day-old seedlings, growing on agar plates, in 10 ml solvent (distilled water or methanol) by shaking for 30 min. Exudates were used at a concentration of 10 μl/ml.

The reporter strains were also lawn plated on SA agar supplemented with X-Gal (40 μg/ml). Alfalfa seedlings, and wooden toothpicks impregnated with exudates, were laid on top of the bacterial lawn, and after 2 days incubation at 28 ºC, plates were inspected microscopically.

RESULTS

Cloning of the *P. fluorescens* F113 *xerD* gene

Two primers derived from the sequence of the *xerD* gene from *P. aeruginosa* PAO1 were used to amplify genomic DNA from *P. fluorescens* F113. A PCR fragment of the expected size (308 bp) was obtained and sequenced. The sequence showed that the amplified fragment corresponded to an internal fragment of the putative *xerD* gene, and it was used to screen an F113 gene library by hybridization. A hybridizing cosmid (pBG1386) was isolated, and primers designed from the sequence of the internal fragment of *xerD* were used to extend the sequence. The full sequence of the F113 *xerD* gene was obtained, and it has been deposited in GenBank (accession no. AY642384).

Analysis of the sequence showed the presence of an ORF encoding a product of 298 aa that showed 79% identity with the *xerD* gene from *P. aeruginosa* PAO1. The ORF also showed a high level of identity (95 %) with the *xerD* gene from the sequenced genome of *P. fluorescens* Pf0-1. The putative *P. fluorescens* F113 XerD sequence shows features typical of site-specific recombinases (Fig. 3.1A), possessing substantial homology with Sss, XerC and XerD proteins, and a

A

```
XerD F113       KSLSPHTLRHAFATHLLNHGADLRVVQMLLGHSDLSTTQIYTHVARARLQDLHAKHHPRG 298
XerD E.coli     EKLSPHVLRHAFATHLLNHGADLRVVQMLLGHSDLSTTQIYTHVATERLRQLHQQHHPRA 298
Sss F113        QNLHPHMLRHSFASHLLESSQDLRAVQELLGHSDIKTTQIYTHLDFQHLATVYDSAHPRA 291
XerC E.coli     NHVHPHKLRHSFATHMLESSGDLRGVQELLGHANLSTTQIYTHLDFQHLASVYDAAHPRA 294
                : : ** ***:**:*:*: . *** ** ****:::.*******:   :*  ::   ***.
```

B

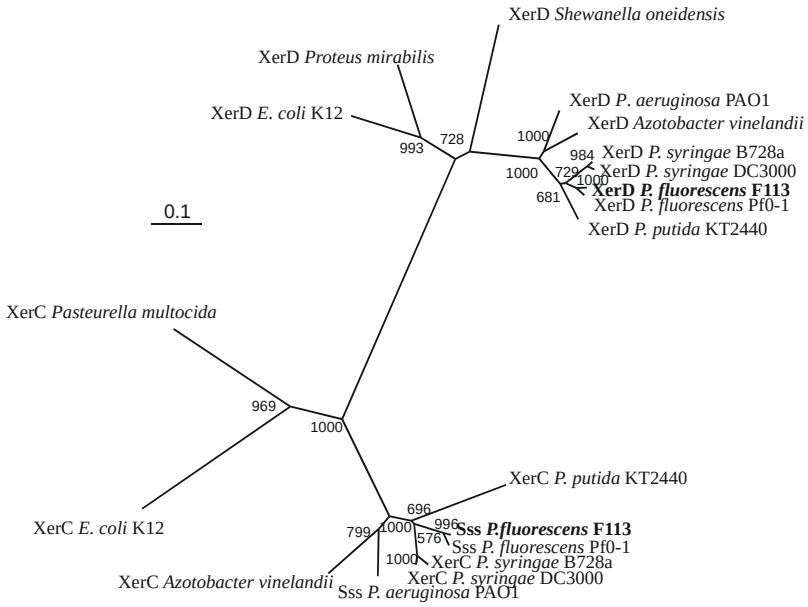

Figure 3.1. Analysis of the *P. fluorescens* F113 *xerD* gene product. (A) Alignment of the C-terminal domain of the putative F113 XerD protein with the F113 Sss and the *E. coli* XerC and XerD proteins. The grey box indicates the conserved domain in the carboxyl terminus of the protein that includes the catalytical tyrosine residue (black box) present in an equivalent position in all these proteins. (B) Cluster analysis (bootstrap NJ tree) of the XerC (Sss) and XerD proteins from several Gram-negative bacteria. F113 are shown in bold.

domain that includes the catalytic tyrosine residue close to the carboxyl terminus, typical of the λ integrase family of recombinases. Identification of this ORF as the F113 *xerD* gene was confirmed by cluster analysis, as the protein grouped with other

recognized XerD proteins from several bacteria, including *E. coli* (Fig. 3.1B). Southern-blot analysis, using internal fragments of F113 *sss* and *xerD*, did not show the presence of additional copies of these genes in the F113 genome (Fig. 3.2).

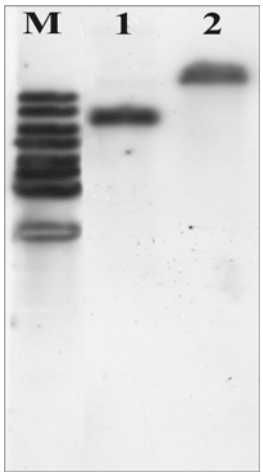

Figure 3.2. Southern blot analysis of *Eco*RI-digested total genomic DNA from *P. fluorescens* F113. Lane 1, *sss* probe; lane 2, *xerD* probe; M, molecular mass markers (1 kb ladder).

Sss and XerD recombinases are implicated in phenotypic variation

F113rif derivatives, carrying mutations in the *sss* and *xerD* genes, were constructed by gene disruption. Despite several efforts, we were unable to construct a stable strain carrying a double mutation affecting both genes, suggesting that the presence of one or other of the recombinases is necessary for cellular viability. The appearance of phenotypic variants with a translucent colony morphology after prolonged laboratory cultivation, and after rhizosphere colonization, was assessed and compared to that of the wild-type strain (Table 3.2). Under prolonged laboratory cultivation conditions, the percentage of phenotypic variants produced by the three strains was very low, but higher in the wild-type strain. However, the appearance of

phenotypic variants after rhizosphere colonization was very different (Table 3.2). While 19% of the colonies recovered from the root tip of plants inoculated with the wild-type were phenotypic variants, less than 5% of the colonies from root tips of plants inoculated with either of the mutants showed a variant morphology. These results show that both recombinases play a role in phenotypic variation during rhizosphere colonization. The ability of both mutants to form phenotypic variants was recovered after complementation by a wild-type copy of the genes cloned in pFAJ1709.

Table 3.2. Percentage of phenotypic variants appearing under laboratory cultivation and rhizosphere colonization conditions

Strain	Phenotypic variants (%)[a]	
	Laboratory cultivation	**Root tip**
F113rif	0.25 ± 0.05	19.00 ± 4.36
F113-sss2	<0.1	4.37 ± 1.21
F113-xerD	<0.1	4.11 ± 2.72
F113rif pBG1457 (*sss* overexpression)	89.50 ± 0.71	97.00 ± 1.83
F113rif pBG1442 (*xerD* overexpression)	11.76 ± 6.95	39.97 ± 3.32
F113-xerD pBG1457	91.68 ± 4.31	69.47 ± 8.56
F113-sss2 pBG1442	<0.1	2.23 ± 0.21

a. Values are means of three experiments ± SD

Derivatives overexpressing the site-specific recombinases were constructed by mobilizing plasmids into F113rif. These plasmids contained either of the genes encoding the recombinases, under the control of the strong *nptII* promoter. The strain overexpressing the *sss* gene (F113rif pBG1457) produced almost 90% variants after prolonged laboratory cultivation, and 97% of the colonies recovered from the root-tip of plants inoculated with this strain were variants (Table 3.2). The strain overexpressing the *xerD* gene (F113rif pBG1442) also produced more variants than the wild-type after prolonged cultivation, although the percentage of variants was much lower than in the case of *sss* overexpression. Similarly, a significant percentage

of the colonies recovered from the root-tip of plants inoculated with F113rif pBG1442 (*xerD* overexpression) showed variant morphology (Table 3.2).

The plasmids overexpressing the genes encoding the site-specific recombinases were also mobilized into the *sss* and *xerD* mutants to generate strains mutated in one of the recombinases, but overexpressing the other. As shown in Table 3.2, overexpression of the *sss* gene in a XerD$^-$ background resulted in a high proportion of phenotypic variants after prolonged cultivation. Therefore, overexpression of *sss* suppressed the phenotype of a *xerD* mutant, indicating that the XerD recombinase is necessary for the wild-type level of phenotypic variation when Sss is produced at a physiological (wild-type) level, but that it plays a marginal role when *sss* is expressed at higher levels. Conversely, the overexpressed *xerD* gene could not suppress the phenotype of the *sss* mutant, as the number of phenotypic variants after prolonged cultivation and rhizosphere passage remained very low. These results show that the Sss recombinase plays a more important role in phenotypic variation than the XerD recombinase, although the latter is necessary for wild-type level of phenotypic variation when *sss* is expressed at a physiological level.

The Gac system is affected by site-specific recombinase activity

The colony morphology of phenotypic variants resembles the morphology of mutants in the GacA/GacS two-component system (Sánchez-Contreras *et al.*, 2002). To test the Gac phenotype of the variants produced by overexpression of both recombinases, 17 variants from the *sss* overexpression experiment, 15 variants from the *xerD* overexpression experiment, and 10 colonies with wild-type morphology obtained from the overexpression experiments were randomly selected and tested for exoprotease and pyoverdine production. *gac* mutants in *P. fluorescens* do not produce exoprotease, and they produce higher levels of the siderophore pyoverdine than the wild-type (Blumer *et al.*, 1999; Duffy & Défago, 2000; Sacherer *et al.*,

1994; Sánchez-Contreras *et al.*, 2002). None of the variants in this study produced a halo in plates containing skim milk, indicating the lack of exoprotease activity (Fig. 3.3B, D), while all the wild-type colonies produced a halo. Similarly, all the variants produced greater fluorescence when plates were observed under UV light, indicating that they produce more pyoverdine (Fig. 3.3A, C). All the wild-type colonies showed a normal level of pyoverdine production. These results indicate that the Gac system is affected by site-specific recombinase activity, and that the variant phenotype is, at least partially, due to mutations in the genes encoding this system.

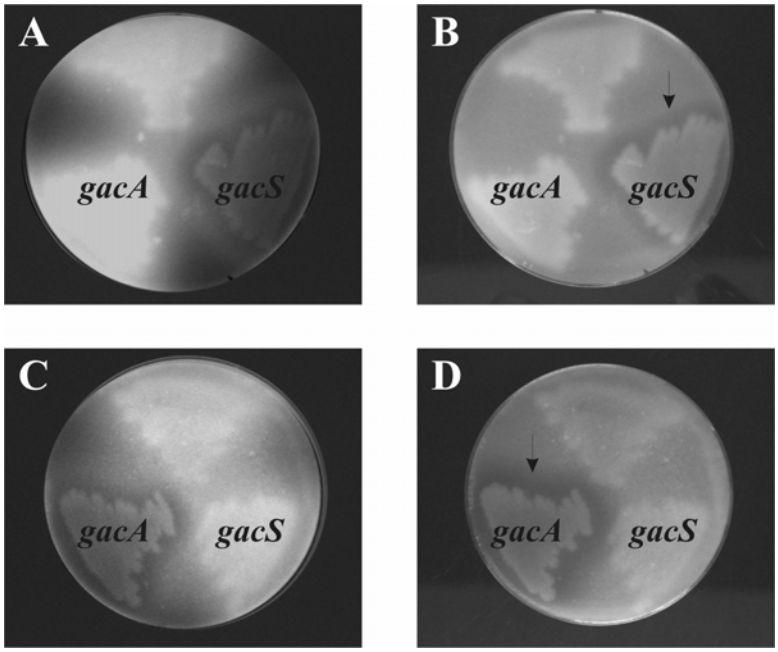

Figure 3.3. Complementation analysis of *P. fluorescens* F113 phenotypic variants. (A, B) A variant complemented by a cloned *gacS* gene. (C, D) A variant complemented by the *gacA* gene. (A, C) Viewed under UV light to test for the amount of pyoverdine produced. (B, D) Viewed under white light to observe the presence of a halo due to exoprotease activity on skim-milk plates. Arrows point to haloes.

A complementation analysis (Fig. 3.3) using the cloned *gacS* gene from *Pseudomonas syringae* pv. *syringae* B728a (Hrabak & Willis, 1992), and the cloned *gacA* gene from *P. fluorescens* CHA0 (Laville *et al.*, 1992), showed that 13 of the variants from the *sss* overexpression experiment, and 11 of the variants from the *xerD* overexpression experiment, were complemented for exoprotease and pyoverdine production by the cloned *gacS* gene (Fig. 3.3A, B). Three variants from each experiment were complemented by the *gacA* gene (Fig. 3.3C, D), and one variant from each experiment was not complemented by either gene (*gacA* or *gacS*). These results suggest that both *gacA* and *gacS* are affected by site-specific recombinase activity.

Sss and XerD are required for competitive rhizosphere colonization

An *sss* mutant of *P. fluorescens* WCS365 has been shown to be defective in tomato root colonization, indicating that the *sss* gene plays an important role in rhizosphere colonization (Dekkers *et al.*, 1998). In order to investigate the putative role of the *xerD* gene in rhizosphere colonization, the *sss* and *xerD* mutants were used to perform competitive rhizosphere colonization experiments with the wild-type strain. Alfalfa seedlings were inoculated with a 1:1 mixture of the wild-type strain and one of the mutants. As shown in Fig. 3.4, both mutants were displaced from the alfalfa root tip by the wild-type strain, indicating that both recombinases are required for competitive colonization.

The *sss* and *xerD* genes are rhizosphere-induced

Disruption of the *sss* and *xerD* genes with an internal fragment of the gene cloned in the plasmid pVIK112 (Kalogeraki & Winans, 1997) generated transcriptional fusions of these genes with a promoterless *lacZ* gene. These strains were used to investigate the regulation of the genes encoding both recombinases.

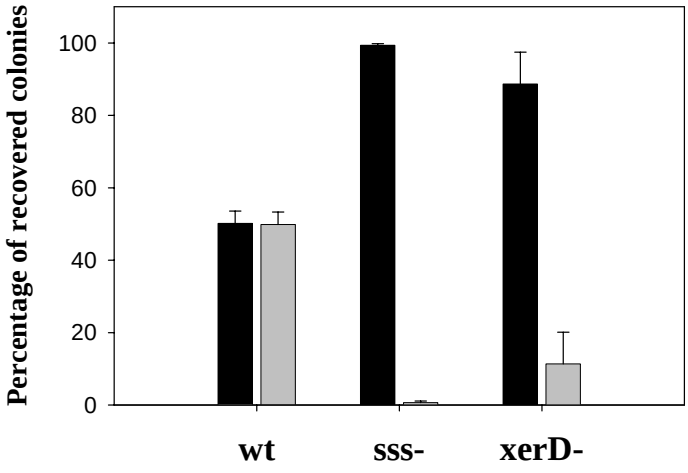

Figure 3.4. Competitive root colonization by *P. fluorescens* F113 and derivatives harbouring mutations in the *sss* and *xerD* genes. A wild-type strain tagged with the same vector used to generate the mutants was used as a competitor for the wild-type strain. The wild-type strain was used as the competitor strain for the mutants. Grey bars represent the percentage of colonies recovered from the test strains, and black bars represent the percentage of colonies recovered from the competitor (wild-type) strain. Results are shown as means ± SD. The mean number of recovered bacteria per gram of root tip was 7.37×10^{6}, the range being from 1.85×10^{6} to 1.77×10^{7}.

As shown in Fig. 3.5A, the expression level of the *sss* gene was low, and was dependent on the growth phase (Fig. 3.5C), being lower during exponential growth and increasing during the stationary phase. When bacteria harbouring this fusion were grown in the presence of aqueous or methanolic root extracts, no differences in expression were observed, indicating that expression of the *sss* gene does not depend on diffusible compounds exuded by the plant root. Fig. 3.5(B, C) shows that the expression level and pattern of the *xerD* gene were similar to those of *sss*, also being independent of compounds present in the root extracts.

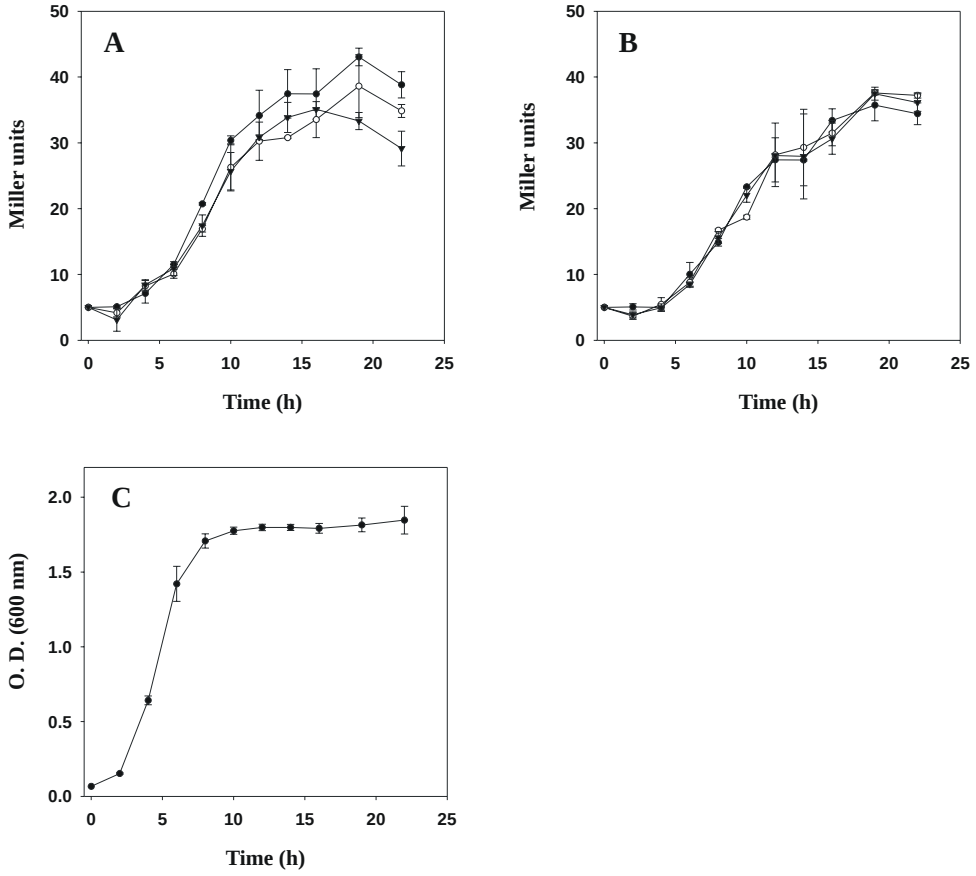

Figure 3.5. Time-course expression (Miller units) of the genes encoding both site-specific recombinases in the absence and presence of root exudates. (A) F113 *sss*::*lac*. (B) F113 *xerD*::*lac*. ● No exudates, ○ aqueous exudates, ▼ methanolic exudates. (C) Growth curve of *P. fluorescens* F113 in LB medium. No differences in growth were observed for the derivatives used in expression experiments in any treatment. Results are shown as means ± standard desviation.

However, both fusions were significantly induced by the presence of the plant root. As observed in Fig. 3.6, cells grown attached to the rhizoplane showed expression of the *lacZ* reporter. Expression was observed only in the bacteria growing in the close vicinity of the root, indicating again that the induction is not due

to any diffusible compound. Furthermore, bacteria growing attached to toothpicks impregnated with aqueous or methanolic extracts did not show induction (data not shown), ruling out that attachment was the only cause of induction. It is therefore possible that a non-diffusible compound present in the root is an inducer of both *sss* and *xerD* expression.

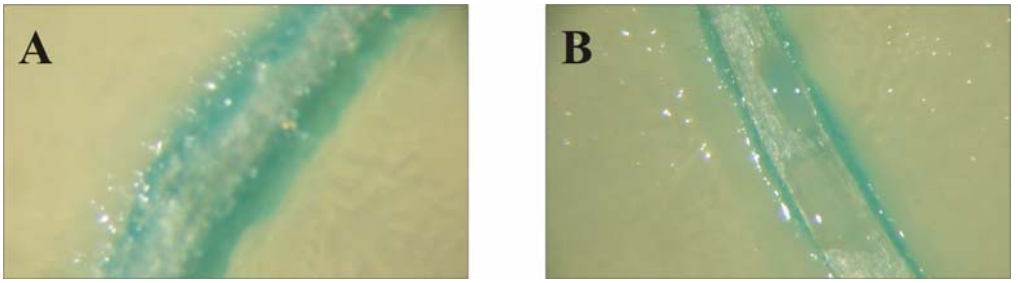

Figure 3.6. Induction of the genes encoding both site-specific recombinases in the alfalfa rhizosphere. β-Galactosidase activity was visualized by the appearance of a blue precipitate in X-Gal containing plates. (A) F113 *sss*::*lac*. (B) F113 *xerD*::*lac*.

DISCUSSION

The Sss recombinase from pseudomonads is an orthologue of XerC (Hofte *et al.*, 1994), and it has been implicated in phenotypic variation (Sánchez-Contreras *et al.*, 2002) and rhizosphere colonization (Dekkers *et al.*, 1998; Sánchez-Contreras *et al.*, 2002). We have sequenced and cloned the *xerD* gene from the rhizosphere colonizer *P. fluorescens* F113, and sequence analysis has shown that it encodes a site-specific recombinase homologous to its *E. coli* counterpart (Fig. 3.1).

A mutant affected in the *xerD* gene showed the same phenotype as an *sss* mutant with regard to phenotypic variation, producing a very low number of phenotypic variants after rhizosphere colonization (Table 3.2). This indicates that not only Sss, but also that XerD is necessary to produce the genetic rearrangements

leading to the appearance of variants. Considering that in *E. coli* the XerC/XerD heterotetramer is necessary for recombination (Ferreira *et al.*, 2003), it seems reasonable to assume that in pseudomonads, Sss and XerD form a heterotetramer responsible for an increase in phenotypic variation. However, we have found differences in the phenotype of strains overproducing either recombinase. Overexpression of either of the genes encoding the two recombinases led to an increase in the proportion of phenotypic variants, although the impact of overproduction of Sss was much higher than overproduction of XerD. Furthermore, overproduction of Sss could overcome the phenotype of a *xerD* mutant, indicating that at high cellular concentration, Sss alone (forming either homodimers or homotetramers) can catalyse recombination events that give rise to variants. Conversely, overexpression of the *xerD* gene was not enough to suppress the *sss* mutation, suggesting that there is a strict requirement of the Sss recombinase for the increase in phenotypic variation. However this interpretation is unlikely because of the increase in the number of phenotypic variants obtained after overproduction of XerD at constant and possibly low levels (see below) of Sss. Furthermore, we have been unable to construct a stable double mutant affecting both recombinases, suggesting that one or other of them is necessary for chromosome segregation after replication. The finding that, in contrast to *E. coli* (Hendricks *et al.*, 2000), single mutants grew at the same rate as the wild-type (data not shown), and with a normal cell morphology, indicate that either of the recombinases can function independently.

Analysis of the genotype of phenotypic variants has shown that all of them carry mutations in the Gac system (Fig. 3.3). Mutations in different variants affected either the *gacS* or the *gacA* genes, although in two variants, the affected gene could not be determined. It is likely that these two variants are affected in both genes, although it is also possible that they are affected in genes acting downstream in the *gac* regulatory cascade. These results could suggest that the *gacA* and *gacS* genes are a target for the site-specific recombinases. However, considering that these genes are located in different parts of the genome, it is unlikely that the activity of the

recombinases would result in the accumulation of diverse mutations in both genes. Therefore, we propose an indirect effect of the site-specific recombinases on the Gac system. The relationship between the Gac system and phenotypic variation has been shown by van den Broek *et al.* (2003). These authors demonstrated that *Pseudomonas* sp. PCL1171 transposon-generated mutants in the *gacS* gene were locked in the variant colony morphology. It has also been shown that a phenotypic variant of *P. fluorescens* F113, isolated from the alfalfa rhizosphere, carried a point mutation in the *gacA* gene (Sánchez-Contreras *et al.*, 2002). In the same report, the few phenotypic variants appearing in the rhizosphere after colonization by an *sss* mutant were defective in the Gac system.

The Sss recombinase has been shown to be essential for competitive root colonization of the potato, radish, wheat and tomato rhizospheres by *P. fluorescens* WCS365 (Dekkers *et al.*, 1998). Here, we show the need of this recombinase for competitive colonization of the alfalfa rhizosphere by another biocontrol strain, *P. fluorescens* F113 (Fig. 3.4). This strain is an efficient colonizer of all plants tested so far, including alfalfa (Villacieros *et al.*, 2003), sugarbeet (Delany *et al.*, 2001; Shanahan *et al.*, 1992), pea (Naseby & Lynch, 1999) and tomato (Simons *et al.*, 1996). It is likely that the activity of site-specific recombinases is a requirement for competitive rhizosphere colonization of a large variety of plants by *P. fluorescens*. We have also shown that an additional recombinase, XerD, is required for competitive rhizosphere colonization (Fig. 3.4). The finding that the proportion of phenotypic variants depends directly on the cellular amount of recombinases, and the higher proportion of variants after rhizosphere passage, indicates that phenotypic variants are selected in the rhizosphere, and it makes a clear link between recombinase activity, phenotypic variation and competence for rhizosphere colonization. It is interesting to note that rhizosphere colonization has been shown to be necessary for biocontrol (Chin-A-Woeng *et al.*, 2000), and that biocontrol traits have been shown to be regulated by phase variation in pseudomonads (van den Broek *et al.*, 2003).

Expression of the genes encoding the two recombinases under laboratory growth conditions was low (Fig. 3.5), as expected for genes whose products might alter the genome structure. The expression pattern showed that the lower level of expression occurred during the exponential phase, probably reflecting the low amounts of recombinases required for chromosome segregation, even in an actively growing population. The expression level was higher during the stationary phase, when cell division is restricted. It is possible that larger amounts of recombinases during this period are related to the stress-induced adaptive mutagenesis that appears during stationary phase in bacteria (Wright, 2004). It is important to note that it has been shown that after prolonged growth, *gacA* and *gacS* mutants accumulate in *P. fluorescens* CHA0 batch cultures (Duffy & Défago, 2000). It is likely that this accumulation of mutants, with a colony morphology and genotype resembling recombinase-induced variants, is partially due to the overproduction of the recombinases during long-term stationary-phase conditions.

Genes encoding the two recombinases are induced in the alfalfa rhizosphere, although no diffusible inducer could be detected in root exudates (Fig. 3.6). Since attachment to toothpicks impregnated with exudates did not induce these genes, it is likely that a non-diffusible inducer present in the plant root is responsible for the increased gene expression. Following a strategy to identify plant-induced genes of *P. syringae*, Marco *et al*. (2003) found that one of the five plant-induced genes detected corresponded to a *xerD* homologue, suggesting that site-specific recombinases might also play a role in pathogenic plant–microbe interactions. Rhizosphere induction also indicates that besides phenotypic selection of variants during rhizosphere colonization, there is also an increase in the production of variants, since we have shown that the proportion of variants is dependent on the expression level of the recombinases. Dekkers *et al*. (2000) have shown that overexpression of the *sss* gene can improve root colonization by different *P. fluorescens* strains. Our results show that the *xerD* gene offers new possibilities to improve rhizosphere colonization and, possibly, biotechnological applications of pseudomonads.

ACKNOWLEDGEMENTS

This work was supported by grants from the European Union (QLK3-CT-2001-00101), the Comunidad Autónoma de Madrid (07 M-0062-2000) and the Spanish Ministry of Science and Technology BIO 2003-03412. M. M. is the recipient of a Ramón y Cajal contract from the Spanish Ministry of Science and Technology.

REFERENCES

Achouak, W., Conrod, S., Cohen, V. & Heulin, T. (2004). Phenotypic variation of *Pseudomonas brassicacearum* as a plant root-colonization strategy. *Mol Plant-Microbe Interact* **17**, 872-879.

Bertani, G. (1951). Studies on Lysogenesis. 1. The Mode of Phage Liberation by Lysogenic *Escherichia coli*. *J Bacteriol* **62**, 293-300.

Blakely, G., Colloms, S., May, G., Burke, M. & Sherratt, D. (1991). *Escherichia coli* XerC recombinase is required for chromosomal segregation at cell division. *New Biol* **3**, 789-798.

Blakely, G., May, G., Mcculloch, R., Arciszewska, L. K., Burke, M., Lovett, S. T. & Sherratt, D. J. (1993). Two related recombinases are required for site-specific recombination at *dif* and *cer* in *Escherichia coli* K12. *Cell* **75**, 351-361.

Blumer, C., Heeb, S., Pessi, G. & Haas, D. (1999). Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. *Proc Natl Acad Sci U S A* **96**, 14073-14078.

Boyer, H. W. & Roulland-Dussoix, D. (1969). A complementation analysis of restriction and modification of DNA in *Escherichia coli*. *J Mol Biol* **41**, 459-472.

Colloms, S. D., Sykora, P., Szatmari, G. & Sherratt, D. J. (1990). Recombination at ColE1 *cer* requires the *Escherichia coli xerC* gene product, a member of the lambda integrase family of site-specific recombinases. *J Bacteriol* **172**, 6973-6980.

Cornet, F., Mortier, I., Patte, J. & Louarn, J. M. (1994). Plasmid pSC101 harbors a recombination site, *psi*, which is able to resolve plasmid multimers and to substitute for the analogous chromosomal *Escherichia coli* site *dif*. *J Bacteriol* **176**, 3188-3195.

Chin-A-Woeng, T. F. C., Bloemberg, G. V., Mulders, I. H. M., Dekkers, L. C. & Lugtenberg, B. J. J. (2000). Root colonization by phenazine-1-carboxamide-producing bacterium *Pseudomonas chlororaphis* PCL1391 is essential for biocontrol of tomato foot and root rot. *Mol Plant-Microbe Interact* **13**, 1340-1345.

Dekkers, L. C., Phoelich, C. C., van der Fits, L. & Lugtenberg, B. J. J. (1998). A site-specific recombinase is required for competitive root colonization by *Pseudomonas fluorescens* WCS365. *Proc Natl Acad Sci U S A* **95**, 7051-7056.

Dekkers, L. C., Mulders, I. H. M., Phoelich, C. C., Chin-A-Woeng, T. F. C., Wijfjes, A. H. M. & Lugtenberg, B. J. J. (2000). The *sss* colonization gene of the tomato-*Fusarium oxysporum F*. sp *radicis-lycopersici* biocontrol strain *Pseudomonas fluorescens* WCS365 can improve root colonization of other wild-type *Pseudomonas* spp. bacteria. *Mol Plant-Microbe Interact* **13**, 1177-1183.

Delany, I. R., Walsh, U. F., Ross, I., Fenton, A. M., Corkery, D. M. & O'Gara, F. (2001). Enhancing the biocontrol efficacy of *Pseudomonas fluorescens* F113 by altering the regulation and production of 2,4-diacetylphloroglucinol. Improved Pseudomonas biocontrol inoculants. *Plant Soil* **232**, 195-205.

Dombrecht, B., Vanderleyden, J. & Michiels, J. (2001). Stable RK2-derived cloning vectors for the analysis of gene expression and gene function in gram-negative bacteria. *Mol Plant-Microbe Interact* **14**, 426-430.

Dove, S. L. & Dorman, C. J. (1994). The site-specific recombination system regulating expression of the type-1 fimbrial subunit gene of *Escherichia coli* is sensitive to changes in DNA supercoiling. *Mol Microbiol* **14**, 975-988.

Duffy, B. K. & Défago, G. (2000). Controlling instability in *gacS-gacA* regulatory genes during inoculant production of *Pseudomonas fluorescens* biocontrol strains. *Appl Environ Microbiol* **66**, 3142-3150.

Fahraeus, G. (1957). The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. *J Gen Microbiol* **16**, 374-381.

Ferreira, H., Butler-Cole, B., Burgin, A., Baker, R., Sherratt, D. J. & Arciszewska, L. K. (2003). Functional analysis of the C-terminal domains of the site-specific recombinases XerC and XerD. *J Mol Biol* **330**, 15-27.

Figurski, D. H. & Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci U S A* **76**, 1648-1652.

Groth, A. C. & Calos, M. P. (2004). Phage integrases: Biology and applications. *J Mol Biol* **335**, 667-678.

Hendricks, E. C., Szerlong, H., Hill, T. & Kuempel, P. (2000). Cell division, guillotining of dimer chromosomes and SOS induction in resolution mutants (*dif*, *xerC* and *xerD*) of *Escherichia coli*. *Mol Microbiol* **36**, 973-981.

Hofte, M., Dong, Q. G., Kourambas, S., Krishnapillai, V., Sherratt, D. & Mergeay, M. (1994). The *sss* gene product, which affects pyoverdin production in *Pseudomonas aeruginosa* 7NSK2, is a site-specific recombinase. *Mol Microbiol* **14**, 1011-1020.

Hrabak, E. M. & Willis, D. K. (1992). The *lemA* gene required for pathogenicity of *Pseudomonas syringae* pv. *syringae* on bean is a member of a family of two-component regulators. *J Bacteriol* **174**, 3011-3020.

Kalogeraki, V. S. & Winans, S. C. (1997). Suicide plasmids containing promoterless reporter genes can simultaneously disrupt and create fusions to target genes of diverse bacteria. *Gene* **188**, 69-75.

Laville, J., Voisard, C., Keel, C., Maurhofer, M., Defago, G. & Haas, D. (1992). Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root-rot of tobacco. *Proc Natl Acad Sci U S A* **89**, 1562-1566.

Marco, M. L., Legac, J. & Lindow, S. E. (2003). Conditional survival as a selection strategy to identify plant-inducible genes of *Pseudomonas syringae*. *Appl Environ Microbiol* **69**, 5793-5801.

Miller, J. H. (1972). Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Naseby, D. C. & Lynch, J. M. (1999). Effects of *Pseudomonas fluorescens* F113 on ecological functions in the pea rhizosphere are dependent on pH. *Microb Ecol* **37**, 248-256.

Sacherer, P., Defago, G. & Haas, D. (1994). Extracellular protease and phospholipase-C are controlled by the global regulatory gene *gacA* in the biocontrol strain *Pseudomonas fluorescens* CHA0. *FEMS Microbiol Lett* **116**, 155-160.

Sambrook, J., Fritsch, E. F. & Maniatics, T. (1989). Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sánchez-Contreras, M., Martín, M., Villacieros, M., O'Gara, F., Bonilla, I. & Rivilla, R. (2002). Phenotypic selection and phase variation occur during alfalfa root colonization by *Pseudomonas fluorescens* F113. *J Bacteriol* **184**, 1587-1596.

Schafer, A., Tauch, A., Jager, W., Kalinowski, J., Thierbach, G. & Puhler, A. (1994). Small mobilizable multipurpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: Selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **145**, 69-73.

Scher, F. M. & Baker, R. (1982). Effects of *Pseudomonas putida* and a synthetic iron chelator on induction of soil suppressiveness to *Fusarium wilt* pathogens. *Phytopathology* **72**, 1567-1573.

Shanahan, P., O´Sullivan, D. J., Simpson, P., Glennon, J. D. & O´Gara, F. (1992). Isolation of 2,4-diacetylphloroglucinol from a fluorescent pseudomonad and investigation of physiological parameters influencing its production. *Appl Environ Microbiol* **58**, 353-358.

Simons, M., van der Bij, A. J., Brand, I., de Weger, L. A., Wijffelman, C. A. & Lugtenberg, B. J. J. (1996). Gnotobiotic system for studying rhizosphere colonization by plant growth-promoting *Pseudomonas* bacteria. *Mol Plant-Microbe Interact* **9**, 600-607.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673-4680.

Tominaga, A., Ikemizu, S. & Enomoto, M. (1991). Site-specific recombinase genes in three *Shigella* subgroups and nucleotide-sequences of a *pinB* gene and an invertible B segment from *Shigella boydii*. *J Bacteriol* **173**, 4079-4087.

van den Broek, D., Chin-A-Woeng, T. F. C., Eijkemans, K., Mulders, I. H. M., Bloemberg, G. V. & Lugtenberg, B. J. J. (2003). Biocontrol traits of *Pseudomonas* spp. are regulated by phase variation. *Mol Plant-Microbe Interact* **16**, 1003-1012.

Villacieros, M., Power, B., Sánchez-Contreras, M. & other authors (2003). Colonization behaviour of *Pseudomonas fluorescens* and *Sinorhizobium meliloti* in the alfalfa (*Medicago sativa*) rhizosphere. *Plant Soil* **251**, 47-54.

Wright, B. E. (2004). Stress-directed adaptive mutations and evolution. *Mol Microbiol* **52**, 643-650.

CAPÍTULO 4

Rhizosphere selection of highly motile phenotypic variants of *Pseudomonas fluorescens* with enhanced competitive colonization ability

ABSTRACT

Phenotypic variants of *Pseudomonas fluorescens* F113 showing a translucent and diffuse colony morphology show enhanced colonization of the alfalfa rhizosphere. We have previously shown that in the biocontrol agent *P. fluorescens* F113, phenotypic variation is mediated by the activity of two site-specific recombinases, Sss and XerD. By overexpressing the genes encoding either of the recombinases, we have now generated a large number of variants (mutants) after selection either by prolonged laboratory cultivation or by rhizosphere passage. All the isolated variants were more motile than the wild-type strain and appear to contain mutations in the *gacA* and/or *gacS* gene. By disrupting these genes and complementation analysis, we have observed that the Gac system regulates swimming motility by a repression pathway. Variants isolated after selection by prolonged cultivation formed a single population with a swimming motility that was equal to the motility of *gac* mutants, being 150% more motile than the wild type. The motility phenotype of these variants was complemented by the cloned *gac* genes. Variants isolated after rhizosphere selection belonged to two different populations: one identical to the population isolated after prolonged cultivation and the other comprising variants that besides a *gac* mutation harbored additional mutations conferring higher motility. Our results show that *gac* mutations are selected both in the stationary phase and during rhizosphere colonization. The enhanced motility phenotype is in turn selected during rhizosphere colonization. Several of these highly motile variants were more competitive than the wild-type strain, displacing it from the root tip within 2 weeks.

INTRODUCTION

Pseudomonas fluorescens F113 is a biocontrol agent isolated from the sugar beet rhizosphere (Delany *et al.*, 2001) and capable of protecting this crop against the pathogenic fungus *Pythium ultimum* (Fenton *et al.*, 1992; Shanahan *et al.*, 1992). In addition, derivatives of this strain with the ability to degrade polychlorinated biphenyls have been constructed by the integration of the *Burkholderia* sp. strain LB400 *bph* operon under the control of different regulatory elements (Brazil *et al.*, 1995; Villacieros *et al.*, 2005). *P. fluorescens* F113 is a good rhizosphere colonizer and can colonize the rhizosphere of different plants such as alfalfa (Villacieros *et al.*, 2003), tomato (Simons *et al.*, 1996), and pea (Naseby & Lynch, 1999).

During alfalfa rhizosphere colonization, F113 undergoes phenotypic variation (Sánchez-Contreras *et al.*, 2002) characterized by the appearance of variants with a translucent and diffuse colony morphology. These variants were more prevalent in distal parts of the root (Achouak *et al.*, 2004; Sánchez-Contreras *et al.*, 2002). Phenotypic variation in this strain appears to be mediated by the activity of two site-specific recombinases, Sss and XerD, since mutants with mutations in either of the genes encoding these recombinases show a severe reduction in the appearance of phenotypic variants after rhizosphere colonization and prolonged laboratory cultivation (Martínez-Granero *et al.*, 2005). Phenotypic variation seems to be an important trait for rhizosphere colonization, and mutants of different *Pseudomonas* strains affected in the *sss* (Dekkers *et al.*, 1998; Martínez-Granero *et al.*, 2005) or *xerD* (Martínez-Granero *et al.*, 2005) genes are severely impaired for competitive rhizosphere colonization. Furthermore, the introduction of additional copies of a cloned *sss* gene improves the colonization abilities of several *Pseudomonas* strains, including F113 (Dekkers *et al.*, 2000).

The overexpression of the *sss* or *xerD* genes in F113 results in an important increase in the production of phenotypic variants after prolonged cultivation in the laboratory or after rhizosphere passage (Martínez-Granero *et al.*, 2005). These variants harboured mutations in the *gacA* and/or the *gacS* genes (Martínez-Granero *et al.*, 2005) encoding a two-component system that regulates the production of multiple secondary metabolites (Laville *et al.*, 1992), including some important for biocontrol, such as exoprotease (Blumer *et al.*, 1999; Siddiqui *et al.*, 2005), pyoverdine (Martínez-Granero *et al.*, 2005), and hydrogen cyanide (Reimmann *et al.*, 1997). Mutations in the Gac system have been shown to accumulate after prolonged cultivation of *P. fluorescens* CHA0 (Duffy & Défago, 2000) and have been reported to be the basis of phenotypic (phase) variation in *Pseudomonas* sp. strain PCL1171 (van den Broek *et al.*, 2005b).

Motility is one of the most important traits for competitive rhizosphere colonization, and mutants incapable of chemotactic motility are among the most defective colonization mutants tested (de Weert *et al.*, 2002; Simons *et al.*, 1996). Even mutants that are still motile, but show decreases compared to the wild-type level of motility, are totally displaced from the root tip in competition experiments (Capdevila *et al.*, 2004). Furthermore, in vivo transcription experiments have shown rhizosphere induction of the *fliO* gene (Ramos-González *et al.*, 2005), implicated in flagellum assembly.

In this study, we have generated a large number of phenotypic variants by overexpression of the genes encoding the site-specific recombinases and selection after prolonged laboratory cultivation and after rhizosphere colonization. Analysis of these variants has shown that enhanced motility is selected in the rhizosphere, while a *gac* mutant phenotype is selected both in the rhizosphere and in the stationary phase. We have also shown that overexpression of site-specific recombinases followed by a single rhizosphere passage allows the isolation of more competitive strains.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions

All the *Pseudomonas fluorescens* strains used here are derivatives of the biocontrol strain F113, which was isolated from the sugar beet rhizosphere (Shanahan *et al.*, 1992). Plasmids were mobilized into *P. fluorescens* by triparental matings using pRK2013 as the helper plasmid (Figurski & Helinski, 1979).

P. fluorescens strains were grown in SA medium (Scher & Baker, 1982) overnight at 28°C; solid growth medium contained 1.5% (w/v) purified agar. *Escherichia coli* strains were grown overnight in Luria-Bertani (LB) medium (Bertani, 1951) at 37°C. Site-specific recombinase genes were overexpressed under the control of the *nptII* promoter in the pFAJ1709 plasmid (Dombrecht *et al.*, 2001), which contains a tetracycline resistance gene. *gacA* and *gacS* mutants were obtained by single homologous recombination of amplified internal fragments from *gacA* and *gacS* genes cloned into the suicide vector pK18*mobsac* (Schafer *et al.*, 1994). For the prolonged laboratory growth experiments, bacteria were grown for 1 week in SA liquid medium supplemented with tetracycline shaking at 28°C, and the cultures were plated in SA tetracycline medium. The following antibiotics were used, when required, at the indicated concentrations: rifampicin, 100 μg/ml; tetracycline, 10 μg/ml for *E. coli* or 70 μg/ml for *P. fluorescens*; and kanamycin, 25 μg/ml for *E. coli* or 50 μg/ml for *P. fluorescens*.

Rhizosphere colonization experiments

Alfalfa seeds were sterilized in 70% ethanol for 2 min and diluted bleach (1:5) for 15 min and rinsed thoroughly with sterile distilled water. Seed vernalization was performed at 4°C overnight, and germination was for 1 day at 28°C. Germinated alfalfa seeds were sown in Leonard jar gnotobiotic systems

(Villacieros *et al.*, 2003) using perlite as the solid substrate and 8 mM KNO_3-supplemented FP (Fahraeus, 1957) as the mineral solution. After 2 days, alfalfa seeds were inoculated with ca. 10^8 cells of the appropriate strains. In competition experiments, strains were inoculated at a 1:1 ratio. Plants were maintained under controlled conditions (16 h in the light at 25°C and 8 h in the dark at 18°C) for 2 weeks. Bacteria were recovered from the rhizosphere by vortexing the root tips (last centimetre of the main root) for 2 min in a tube containing 5 ml of 0.9% NaCl and plating the appropriate dilutions on SA plates. Every experiment was performed three times with three replicates each time, and every replicate contained at least 20 plants.

Swimming assays

SA medium plates containing 0.3% purified agar were used to test swimming abilities. Swimming assays were done with variants obtained after independent overexpression of site-specific recombinases (*sss* and *xerD*) either after long laboratory culture conditions or after rhizosphere colonization. The selected variants were cured from the site-specific recombinase overexpression plasmids after several platings without selection. The swimming ability of these derivatives was tested in comparison with the wild-type strain. Complementation assays were done using the plasmid-cloned *gacA* gene from *Pseudomonas fluorescens* CHA0 (pME3066) (Laville *et al.*, 1992) (tetracycline resistant) and the *gacS* gene from *Pseudomonas syringae* (pEMH97) (Hrabak & Willis, 1992) (tetracycline resistant). A wild-type strain harbouring the empty pFAJ1709 plasmid was used as a control. The cells from exponentially growing cultures were inoculated in the middle of the plate in triplicate using a toothpick. Swimming haloes were measured after 18, 24, and 42 h of inoculation. Every assay was done at least three times.

Statistical analysis

Statistical analysis was done with Sigma Plot 4 and SigmaStat 3.1 software. The Lorentzian (three parameters) equation was used to represent motility distribution, and the Kolmogorov-Smirnov test was used to determine the normality ($P > 0.05$) of the estimated underlying population.

RESULTS

Phenotypic variants of *P. fluorescens* F113 are hypermotile

Overexpression of the genes encoding the Sss and XerD site-specific recombinases in *P. fluorescens* F113 results in the generation of a large number of phenotypic variants after both prolonged laboratory cultivation and rhizosphere colonization (Martínez-Granero *et al.*, 2005). Plasmids pBG1457 (pnptII::*sss*) and pBG1442 (pnptII::*xerD*) (Martínez-Granero *et al.*, 2005) were independently introduced into strain F113 cells that were either cultivated in SA medium or applied to alfalfa seedlings. After 1 week of cultivation or 2 weeks of colonization, cells were plated on SA and phenotypic variants, characterized by a translucent colony morphology (Sánchez-Contreras *et al.*, 2002), were randomly picked. One hundred fifty variants from the culture experiments and 200 variants from the rhizosphere experiments were isolated and tested for swimming motility. All the isolated phenotypic variants showed enhanced motility when compared with the wild-type strain, ranging from 120% to 300% (considering 100% the motility of the wild-type strain). These results indicate that hypermotility is a general trait of phenotypic variants.

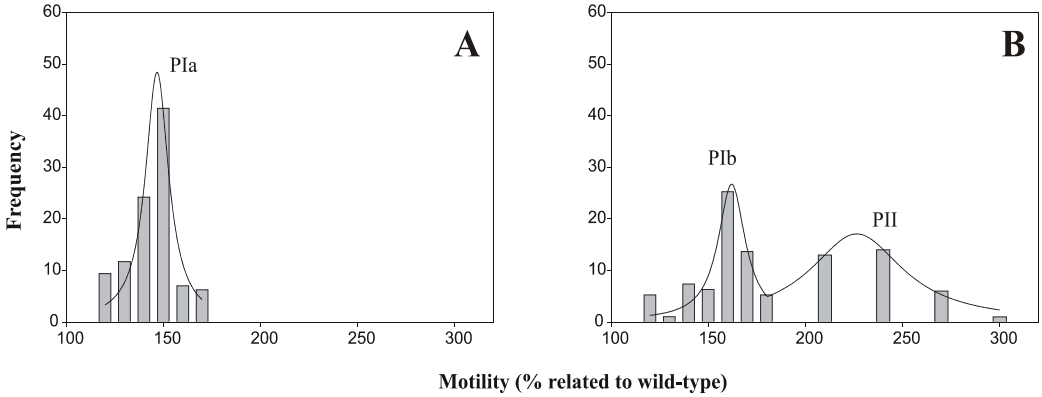

Figure 4.1. Frequency distribution of motility phenotypes among phenotypic variants produced after overexpression of the *sss* or *xerD* gene and selection by prolonged cultivation in SA medium (A) or alfalfa rhizosphere passage (B). Swimming haloes in SA plates were measured and were assigned to frequency intervals. Bar diagrams were shown to fit a normal distribution for the three detected peaks, PIa, PIb, and PII.

Rhizosphere selects hypermotile variants

Figure 4.1 shows the distribution of motility phenotypes among the variants isolated from liquid culture (Fig. 4.1A) and rhizosphere (Fig. 4.1B). All variants were classified in intervals according to their swimming halo diameter compared with the wild-type strain. Clear differences can be observed between variants isolated from liquid culture and those isolated from the rhizosphere. The variants isolated from liquid culture are all grouped in a narrow peak (PIa) indicating that they form a single population with an average motility of 150%. The variants isolated from the rhizosphere showed a wider distribution, and they are grouped in two peaks, suggesting the presence of more than one population. The first peak (PIb) is also narrow and overlaps with the peak observed in the variants isolated from liquid culture, suggesting that they represent the same population. The second peak observed in the variants isolated from the rhizosphere (PII) is wider and

represents one or several populations not appearing in liquid culture. Statistical analysis showed that the frequencies within each of the peaks follow a normal distribution ($P = 0.187$ for peak PIa, $P = 0.153$ for peak PIb, and $P > 0.200$ for peak PII). The *t* test and chi-square test showed that the peak obtained from liquid culture is not different from the first peak obtained from rhizosphere ($P < 0.001$), but both peaks are significantly different from peak PII ($P < 0.001$).

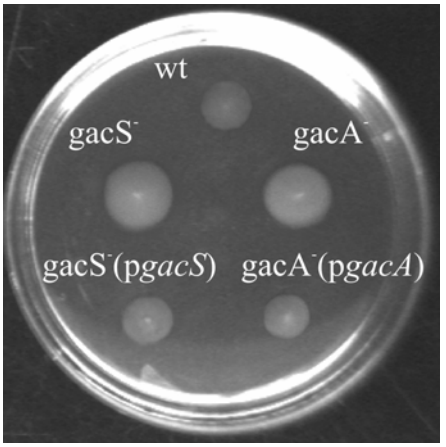

Figure 4.2. Swimming motility haloes produced by *P. fluorescens* F113 and derivatives harbouring insertion mutations in the *gacA* and *gacS* genes. Complementation analysis was performed with mutant strains containing the cloned *gac* genes (p*gacA*/*S*). The empty vectors (not shown) had no effect on halo formation. Cells were inoculated on SA plates and observed after 24 h. wt, wild-type.

Peaks PIa and PIb variants are Gac mutants

Since most variants presented a mutation in the *gacA/gacS* genes (Martínez-Granero *et al.*, 2005), F113 derivatives harbouring mutations in either gene were constructed. As shown in Fig. 4.2, *gacA* and *gacS* mutants are more motile than the wild-type strain, forming a swimming halo of 147% compared with the 100% halo of the wild-type strain. Introduction of the cloned *gacA* and *gacS*

genes complemented the hypermotility phenotype of the mutants, restoring motility to the wild-type level. These results clearly show that the Gac system modulates motility by a repression pathway.

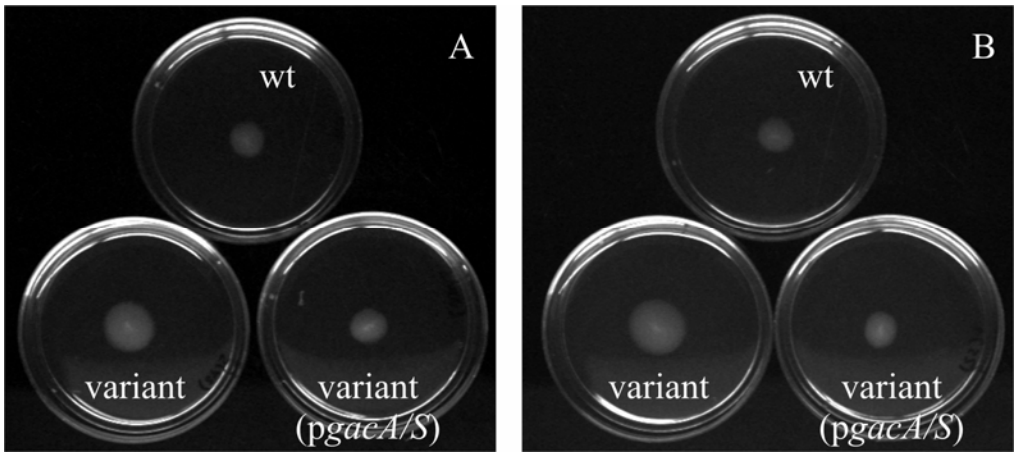

Figure 4.3. Swimming motility phenotype and complementation analysis of phenotypic variants belonging to peaks PIa (A) and PIb (B), respectively, isolated after prolonged cultivation and rhizosphere passage. The figure presents a typical complementation experiment in which either the cloned *gacA* or *gacS* (p*gacA*/*S*) genes totally complemented the motility phenotype of the phenotypic variants. wt, wild-type.

Thirty-two variants from peak PIa were selected for complementation analysis, plasmids expressing either the *gacA* or *gacS* genes (Martínez-Granero *et al.*, 2005) were introduced by triparental mating, and the motility was compared to those of the wild-type strain and the non-complemented variant. Wild-type motility was restored in 30 variants: 24 by the cloned *gacS* gene and 6 by the *gacA* gene. The remaining two variants were not complemented by any gene and are likely to be affected in both genes. Figure 4.3A shows a typical complementation of one of these variants. The same experiment was performed with 51 variants from peak PIb. Wild-type motility was restored in 41 of the variants: 34 by *gacS* and 7 by *gacA*. Figure 4.3B shows the results of one of the complementation assays. These results

indicate that the first peak population is affected in the Gac system and that *gac* mutants are selected both during the late stationary phase and during rhizosphere colonization.

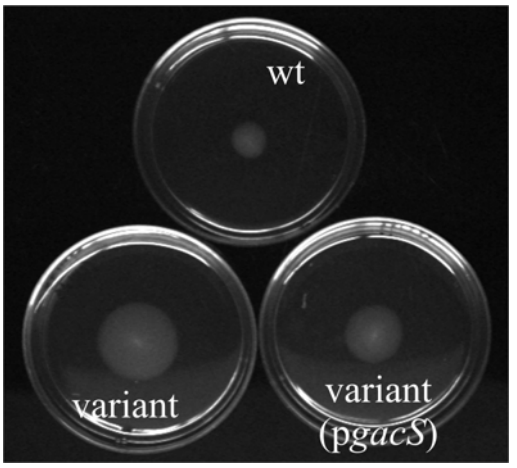

Figure 4.4. Swimming motility phenotype of phenotypic variants belonging to peak PII isolated after rhizosphere selection. The figure presents a typical complementation experiment in which a cloned *gacS* gene (p*gacS*) complemented only partially the motility phenotype of the phenotypic variants. wt, wild-type.

Peak PII variants harbour other mutations besides Gac mutations

Complementation analysis of 14 variants from peak PII showed that none of them had motility restored to wild-type levels by either of the *gac* genes. However, as shown in Fig. 4.4, the motility phenotype of all the variants tested was partially complemented by the cloned *gacS* gene. These variants showed a halo of 229% ± 12% compared to the wild-type level (100%); no significant differences were found when complementation was done with the *gacA* gene (204% ± 13%). Conversely, the cloned *gacS* gene partially complemented the motility phenotype (137% ± 14%). These results indicate that these rhizosphere-isolated variants

harbour mutations in the *gacS* gene and at least one additional mutation leading to increased motility. Therefore, the rhizosphere selects for these hypermotile variants with multiple mutations that are not selected or generated by the late stationary phase.

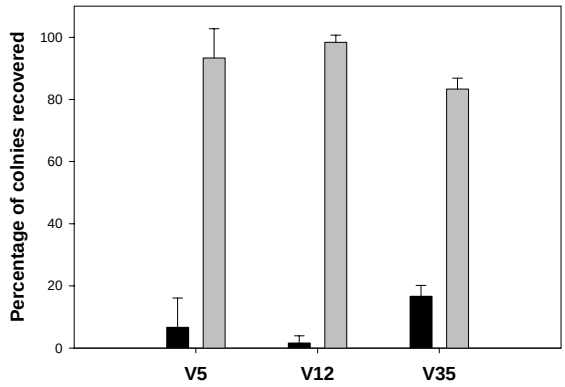

Figure 4.5. Competitive root colonization by *P. fluorescens* F113 and phenotypic variants V5, V12, and V35 isolated after rhizosphere colonization. The wild-type strain was used as the competitor for the phenotypic variants. Grey bars represent the percentage of colonies recovered from the tested strains; black bars represent the percentage of colonies recovered from the competitor (wild-type) strain. Arithmetic means and standard deviations are presented.

Hypermotile variants from rhizosphere peak PII are more competitive than the wild-type strain for rhizosphere colonization

The three variants isolated from the rhizosphere and belonging to peak PII (V5, V12, and V35) that showed the highest motility were selected for competitive colonization experiments. The variants were cured for the plasmids expressing the site-specific recombinases, since it has been previously shown that *P. fluorescens* F113 overexpressing the *sss* gene shows enhanced competitive colonization (Dekkers *et al.*, 2000). As shown in Fig. 4.5, the three variants were more

competitive than the wild-type strain, being able to displace wild-type *P. fluorescens* F113 from the last centimetre of the root within 2 weeks.

DISCUSSION

Motility is an important trait for competitive rhizosphere colonization. Here we show that the rhizosphere selects for hypermotile mutants and that these mutants present enhanced competitive colonization. The fact that all the phenotypic variants isolated from the rhizosphere are more motile than the wild-type strain highlights the importance of motility for rhizosphere colonization.

Phenotypic (phase) variation has been frequently associated with rhizosphere colonization by pseudomonads. It has been shown that phenotypic variants arise during alfalfa root colonization by *P. fluorescens* (Sánchez-Contreras *et al.*, 2002) and during *Arabidopsis thaliana* root colonization by *Pseudomonas brassicacearum* (Achouak *et al.*, 2004), indicating that this might be a general fact of rhizosphere colonization by these bacteria. We have previously shown that overexpression of either of two rhizosphere-induced genes, *sss* and *xerD*, encoding site-specific recombinases accounts for a large increase in the number of variants obtained, especially after rhizosphere colonization (Martínez-Granero *et al.*, 2005). Considering that a *P. fluorescens* mutant affected in the *sss* gene is impaired in rhizosphere colonization of a variety of crops (Dekkers *et al.*, 1998) and that the rhizosphere colonization ability of several pseudomonads can be increased by ectopic expression of this gene (Dekkers *et al.*, 2000), it can be concluded that phenotypic variation is an important trait for rhizosphere competitive colonization, especially after the finding, reported here, that all the variants show enhanced motility.

We have made use of the increase in the number of variants obtained after overexpression of either of the genes encoding site-specific recombinases

(Martínez-Granero *et al.*, 2005) to analyze a large number of phenotypic variants. Besides enhanced motility, we have observed that a vast majority of these variants are affected in the Gac system, a two-component system that regulates multiple traits, including the formation of a variety of secondary metabolites (Laville *et al.*, 1992). The results presented here show that the Gac system also regulates swimming motility through a repression pathway. We are currently investigating this regulatory circuit, since for most traits, the Gac system acts as a posttranscriptional activator (Reimmann *et al.*, 1997). Our previous results (Martínez-Granero *et al.*, 2005) showed a linkage between phenotypic variation and the Gac system that we have confirmed here by analyzing a larger number of phenotypic variants. It is interesting to note that different types of mutations in the *gacA* and -*S* genes (point mutations, tandem repeats, insertions, inversions, and short and long deletions) have been proposed as a mechanism for phenotypic (phase) variation in *Pseudomonas* sp. strain PCL1171 (van den Broek *et al.*, 2005a; van den Broek *et al.*, 2005b; van den Broek *et al.*, 2005c). The fact that different types of mutations are observed in these genes (van den Broek *et al.*, 2005b), together with the locations of the *gacA* and *gacS* genes in different parts of the genome and the observation reported here of a mutation bias toward the larger *gacS* open reading frame, points out that the role of site-specific recombination in the appearance of these mutants is indirect and, according to van den Broek *et al.* (2005a; 2005c), is possibly related to the activity of the *mutS* and *rpoS* genes.

We have observed a different pattern of hypermotile variants between stationary-phase cultures and rhizosphere colonization. All the phenotypic variants isolated after prolonged laboratory cultivation show a motility that is about 150% of the wild-type motility. All these phenotypic variants cluster in a narrow peak when a frequency distribution of motility is plotted, suggesting that they all belong to the same population. In fact, the increase in motility corresponds to the increase observed for *gac* mutants (Fig. 4.2), and their motility phenotype is complemented by the cloned *gac* genes (Fig. 4.3A), indicating that *gac* mutants are selected during

the stationary phase. This complementation analysis has been done with the cloned *gacA* gene from *P. fluorescens* CHA0 (Laville *et al.*, 1992), being the GacA protein from strain CHA0 97% identical to F113 GacA. To complement the *gacS* mutants, we have used the cloned gene from *P. syringae* (Hrabak & Willis, 1992). This GacS protein presents 78% identity and 88% homology to its *P. fluorescens* F113 counterpart (data not shown). The total complementation of both insertion mutants with these heterologous genes shows that they are functionally equivalent to the F113 genes. Duffy and Defago (2000) showed that *gac* mutants arose during prolonged cultivation of *P. fluorescens* CHA0. The characterization of several of these mutants showed that they were caused by independent point and deletion mutations in different parts of the *gacA* gene (Bull *et al.*, 2001). It is possible that induction of genes encoding site-specific recombinases (Martínez-Granero *et al.*, 2005) is a mechanism for generating diversity, *gac* mutants being selected because of their increased fitness under stationary-phase conditions.

The phenotypic variants isolated after rhizosphere colonization clustered in two groups. The first group (peak PIb) seems to be identical to the variants isolated after prolonged cultivation and therefore correspond to *gac* mutants. The cloned *gac* genes were able to complement the motility phenotype of most of these variants (Fig. 4.3B), confirming that they are mutated in the Gac system. Since the *sss* and *xerD* genes are also induced in the rhizosphere (Martínez-Granero *et al.*, 2005), a similar mechanism as for stationary-phase variation can be suggested, with *gac* mutants being selected because of their increased fitness in the rhizosphere. Chancey *et al*. (2002) previously showed that *gac* mutants of *Pseudomonas aureofaciens* arise in the wheat rhizosphere in soil microcosms, comprising up to 36% of the recovered cells. They also showed that *gac* mutants did not appear through a conserved mutational mechanism, in agreement with the results obtained by others (Bull *et al.*, 2001; van den Broek *et al.*, 2005b) in laboratory cultivation of different pseudomonads. It is interesting to note that although *gac* mutants do not seem to be impaired in rhizosphere persistence and colonization in soil microcosms

(Natsch *et al.*, 1994; Schmidt-Eisenlohr *et al.*, 2003), they do not displace wild-type populations (Chancey *et al.*, 2002).

The second group of phenotypic variants isolated from the rhizosphere (peak PII) form a wider peak, with higher motility than *gac* mutants. It is important to note that all the tested variants from this group harbour a mutation that is partially complemented by the cloned *gacS* gene and are therefore affected in the Gac system. The higher motility of these variants indicates that besides the *gac* mutation, they harbour additional mutations that derepress motility. It cannot be excluded that other phenotypes are also selected in the rhizosphere. The width of the peak probably means that it is a mixed population with several additive mutations, suggesting the presence of multiple regulatory circuits repressing motility. Our preliminary unpublished results that show that about 1% of transposon insertions result in more motile mutants support this hypothesis that implies that motility in *P. fluorescens* is severely limited. We are currently investigating the nature of these mutations in order to clarify the genetic constraints responsible for regulating motility. The isolation of these hypermotile variants from the rhizosphere and not from prolonged laboratory cultivation indicates that the enhanced motility phenotype is advantageous during rhizosphere colonization and is therefore selected under these conditions. To strengthen this observation, we have shown here that several of these hypermotile variants are more competitive than the wild-type strain, displacing it from the root tip (Fig. 4.5). Dekkers *et al.* (2000) showed that the competitive colonization ability of several pseudomonads can be increased by overexpression of the *sss* gene. In the experiments reported here, the phenotypic variants were cured of the plasmids overexpressing the site-specific recombinases, indicating that the enhancement of competitive colonization is not directly related to the production of the recombinases but to the mutations generated after site-specific recombinase activity and subsequent rhizosphere selection. The fact that *gac* mutants are not more competitive than the wild type (Chancey *et al.*, 2002) points out that these additional mutations are the basis for increased competitiveness.

The results presented here provide a new method to generate more competitive strains for rhizosphere colonization. Since efficient rhizosphere colonization is a requirement for biotechnological applications in biocontrol and rhizoremediation (Chin-A-Woeng *et al.*, 2000; Kuiper *et al.*, 2001), engineering of competitiveness can be rendered in more effective strains. It has been previously shown that after three enrichment cycles in the rhizosphere, a *P. fluorescens* strain harboring a mutation in the *mutY* gene showed enhanced competitive colonization (de Weert *et al.*, 2004). Since the MutY protein is implicated in DNA repair and a mutant is therefore prone to accumulate mutations that can be selected by the rhizosphere environment, a similar mechanism can be inferred for the overexpression of the recombinases and selection of hypermotile strains after a single rhizosphere passage. It is important to note that after curing the plasmids encoding the recombinases, the resulting more-competitive strains are genetically stable. Although *gac* mutations affect several traits important for biocontrol, the finding that additional mutations conferring increased motility result in enhanced competitive colonization could allow, through uncoupling of this trait from the Gac system, the design of improved biocontrol strains.

ACKNOWLEDGMENTS

We would like to thank Miguel Redondo-Nieto for helping with the preparation of the manuscript. This work was funded by the Spanish Ministry of Science and Education grant BIO2003-03412 and Comunidad de Madrid grant GR/AMB/0084/2004 and by the Research Program MICROAMBIENTE-CM from Comunidad de Madrid. M. Martin was the recipient of a Ramón y Cajal contract from the Spanish Ministry of Science and Education.

REFERENCES

Achouak, W., Conrod, S., Cohen, V. & Heulin, T. (2004). Phenotypic variation of *Pseudomonas brassicacearum* as a plant root-colonization strategy. *Mol Plant-Microbe Interact* **17**, 872-879.

Bertani, G. (1951). Studies on Lysogenesis. 1. The Mode of Phage Liberation by Lysogenic *Escherichia coli*. *J Bacteriol* **62**, 293-300.

Blumer, C., Heeb, S., Pessi, G. & Haas, D. (1999). Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. *Proc Natl Acad Sci U S A* **96**, 14073-14078.

Brazil, G. M., Kenefick, L., Callanan, M., Haro, A., de Lorenzo, V., Dowling, D. N. & O´Gara, F. (1995). Construction of a rhizosphere pseudomonad with potential to degrade polychlorinated biphenyls and detection of *bph* gene expression in the rhizosphere. *Appl Environ Microbiol* **61**, 1946-1952.

Bull, C. T., Duffy, B., Voisard, C., Defago, G., Keel, C. & Haas, D. (2001). Characterization of spontaneous gacS and gacA regulatory mutants of *Pseudomonas fluorescens* biocontrol strain CHA0. *Antonie Van Leeuwenhoek* **79**, 327-336.

Capdevila, S., Martínez-Granero, F. M., Sánchez-Contreras, M., Rivilla, R. & Martín, M. (2004). Analysis of *Pseudomonas fluorescens* F113 genes implicated in flagellar filament synthesis and their role in competitive root colonization. *Microbiol* **150**, 3889-3897.

Chancey, S. T., Wood, D. W., Pierson, E. A. & Pierson, L. S. (2002). Survival of GacS/GacA mutants of the biological control bacterium *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere. *Appl Environ Microbiol* **68**, 3308-3314.

Chin-A-Woeng, T. F. C., Bloemberg, G. V., Mulders, I. H. M., Dekkers, L. C. & Lugtenberg, B. J. J. (2000). Root colonization by phenazine-1-carboxamide-producing bacterium *Pseudomonas chlororaphis* PCL1391 is essential for biocontrol of tomato foot and root rot. *Mol Plant-Microbe Interact* **13**, 1340-1345.

de Weert, S., Vermeiren, H., Mulders, I. H. M., Kuiper, I., Hendrickx, N., Bloemberg, G. V., Vanderleyden, J., de Mot, R. & Lugtenberg, B. J. J. (2002). Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by *Pseudomonas fluorescens*. *Mol Plant-Microbe Interact* **15**, 1173-1180.

de Weert, S., Dekkers, L. C., Kuiper, I., Bloemberg, G. V. & Lugtenberg, B. J. J. (2004). Generation of enhanced competitive root-tip-colonizing *Pseudomonas* bacteria through accelerated evolution. *J Bacteriol* **186**, 3153-3159.

Dekkers, L. C., Phoelich, C. C., van der Fits, L. & Lugtenberg, B. J. J. (1998). A site-specific recombinase is required for competitive root colonization by *Pseudomonas fluorescens* WCS365. *Proc Natl Acad Sci U S A* **95**, 7051-7056.

Dekkers, L. C., Mulders, I. H. M., Phoelich, C. C., Chin-A-Woeng, T. F. C., Wijfjes, A. H. M. & Lugtenberg, B. J. J. (2000). The *sss* colonization gene of the tomato-*Fusarium oxysporum F*. sp *radicis-lycopersici* biocontrol strain *Pseudomonas fluorescens* WCS365 can improve root colonization of other wild-type *Pseudomonas* spp. bacteria. *Mol Plant-Microbe Interact* **13**, 1177-1183.

Delany, I. R., Walsh, U. F., Ross, I., Fenton, A. M., Corkery, D. M. & O'Gara, F. (2001). Enhancing the biocontrol efficacy of *Pseudomonas fluorescens* F113 by altering the regulation and production of 2,4-diacetylphloroglucinol. Improved Pseudomonas biocontrol inoculants. *Plant Soil* **232**, 195-205.

Dombrecht, B., Vanderleyden, J. & Michiels, J. (2001). Stable RK2-derived cloning vectors for the analysis of gene expression and gene function in gram-negative bacteria. *Mol Plant-Microbe Interact* **14**, 426-430.

Duffy, B. K. & Défago, G. (2000). Controlling instability in *gacS-gacA* regulatory genes during inoculant production of *Pseudomonas fluorescens* biocontrol strains. *Appl Environ Microbiol* **66**, 3142-3150.

Fahraeus, G. (1957). The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. *J Gen Microbiol* **16**, 374-381.

Fenton, A. M., Stephens, P. M., Crowley, J., O´Callaghan, M. & O´Gara, F. (1992). Exploitation of gene(s) involved in 2,4-diacetylphloroglucinol biosynthesis to confer a new biocontrol capability to a *Pseudomonas* strain. *Appl Environ Microbiol* **58**, 3873-3878.

Figurski, D. H. & Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci U S A* **76**, 1648-1652.

Hrabak, E. M. & Willis, D. K. (1992). The *lemA* gene required for pathogenicity of *Pseudomonas syringae* pv. *syringae* on bean is a member of a family of two-component regulators. *J Bacteriol* **174**, 3011-3020.

Kuiper, I., Bloemberg, G. V. & Lugtenberg, B. J. J. (2001). Selection of a plant-bacterium pair as a novel tool for rhizostimulation of polycyclic aromatic hydrocarbon-degrading bacteria. *Mol Plant-Microbe Interact* **14**, 1197-1205.

Laville, J., Voisard, C., Keel, C., Maurhofer, M., Defago, G. & Haas, D. (1992). Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root-rot of tobacco. *Proc Natl Acad Sci U S A* **89**, 1562-1566.

Martínez-Granero, F., Capdevila, S., Sánchez-Contreras, M., Martín, M. & Rivilla, R. (2005). Two site-specific recombinases are implicated in phenotypic variation and competitive rhizosphere colonization in *Pseudomonas fluorescens*. *Microbiol* **151**, 975-983.

Naseby, D. C. & Lynch, J. M. (1999). Effects of *Pseudomonas fluorescens* F113 on ecological functions in the pea rhizosphere are dependent on pH. *Microb Ecol* **37**, 248-256.

Natsch, A., Keel, C., Pfirter, H. A., Haas, D. & Defago, G. (1994). Contribution of the global regulator gene *gacA* to persistence and dissemination of *Pseudomonas fluorescens* biocontrol strain CHAO introduced into soil microcosms. *Appl Environ Microbiol* **60**, 2553-2560.

Ramos-González, M. I., Campos, M. J. & Ramos, J. L. (2005). Analysis of *Pseudomonas putida* KT2440 gene expression in the maize rhizosphere: In vivo expression technology capture and identification of root-activated promoters. *J Bacteriol* **187**, 5504-5504.

Reimmann, C., Beyeler, M., Latifi, A., Winteler, H., Foglino, M., Lazdunski, A. & Haas, D. (1997). The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer N-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. *Mol Microbiol* **24**, 309-319.

Sánchez-Contreras, M., Martín, M., Villacieros, M., O'Gara, F., Bonilla, I. & Rivilla, R. (2002). Phenotypic selection and phase variation occur during alfalfa root colonization by *Pseudomonas fluorescens* F113. *J Bacteriol* **184**, 1587-1596.

Schafer, A., Tauch, A., Jager, W., Kalinowski, J., Thierbach, G. & Puhler, A. (1994). Small mobilizable multipurpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: Selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **145**, 69-73.

Scher, F. M. & Baker, R. (1982). Effects of *Pseudomonas putida* and a synthetic iron chelator on induction of soil suppressiveness to *Fusarium wilt* pathogens. *Phytopathology* **72**, 1567-1573.

Schmidt-Eisenlohr, H., Gast, A. & Baron, C. (2003). Inactivation of *gacS* does not affect the competitiveness of *Pseudomonas chlororaphis* in the *Arabidopsis thaliana* rhizosphere. *Appl Environ Microbiol* **69**, 1817-1826.

Shanahan, P., O´Sullivan, D. J., Simpson, P., Glennon, J. D. & O´Gara, F. (1992). Isolation of 2,4-diacetylphloroglucinol from a fluorescent pseudomonad and investigation of physiological parameters influencing its production. *Appl Environ Microbiol* **58**, 353-358.

Siddiqui, I. A., Haas, D. & Heeb, S. (2005). Extracellular protease of *Pseudomonas fluorescens* CHA0, a biocontrol factor with activity against the root-knot nematode *Meloidogyne incognita*. *Appl Environ Microbiol* **71**, 5646-5649.

Simons, M., van der Bij, A. J., Brand, I., de Weger, L. A., Wijffelman, C. A. & Lugtenberg, B. J. J. (1996). Gnotobiotic system for studying rhizosphere colonization by plant growth-promoting *Pseudomonas* bacteria. *Mol Plant-Microbe Interact* **9**, 600-607.

van den Broek, D., Bloemberg, G. V. & Lugtenberg, B. (2005a). The role of phenotypic variation in rhizosphere *Pseudomonas* bacteria. *Environ Microbiol* **7**, 1686-1697.

van den Broek, D., Chin-A-Woeng, T. F. C., Bloemberg, G. V. & Lugtenberg, B. J. J. (2005b). Molecular nature of spontaneous modifications in *gacS* which cause colony phase variation in *Pseudomonas* sp strain PCL1171. *J Bacteriol* **187**, 593-600.

van den Broek, D., Chin-A-Woeng, T. F. C., Bloemberg, G. V. & Lugtenberg, B. J. J. (2005c). Role of RpoS and MutS in phase variation of *Pseudomonas* sp PCL1171. *Microbiol* **151**, 1403-1408.

Villacieros, M., Power, B., Sánchez-Contreras, M. & other authors (2003). Colonization behaviour of *Pseudomonas fluorescens* and *Sinorhizobium meliloti* in the alfalfa (*Medicago sativa*) rhizosphere. *Plant Soil* **251**, 47-54.

Villacieros, M., Whelan, C., Mackova, M. & other authors (2005). Polychlorinated biphenyl rhizoremediation by *Pseudomonas fluorescens* F113 derivatives, using a *Sinorhizobium meliloti* nod system to drive *bph* gene expression. *Appl Environ Microbiol* **71**, 2687-2694.

CAPÍTULO 5

The Gac-Rsm cascade negatively regulates swimming motility and pyoverdine production in *Pseudomonas fluorescens*

ABSTRACT

In many Gram-negative bacteria, the GacA/GacS system positively controls the expression of secondary metabolites and exoenzymes required both for virulence and for biocontrol. This regulatory cascade functions at a posttranscriptional level involving RNA-binding proteins as a key regulatory element. We have previously shown that in the biocontrol agent *P. fluorescens* F113, swimming motility and pyoverdine production are negatively regulated by the Gac system. Here, it is shown that in the model strain *P. fluorescens* Pf0-1 swimming motility and pyoverdine production are also under the negative control of the Gac system. Overexpression of either RNA-binding proteins RsmA or RsmE mimicked the phenotype of a *gac* mutant, indicating that positive and negative regulations occur through the same pathway. The enhanced motility phenotype showed by both *gac* mutants and Rsm-overexpressing strains, is due to an increase in the level of transcription of the *fleQ* and *fliC* genes. However, a translational fusion of the *vfr* gene and swimming assays demostrated that unlike the negative role of Vfr in *fleQ* gene transcription in *P. aeruginosa*, Gac-mediated downregulation of *fleQ* gene in *P. fluorescens* F113 is independent of the regulatory protein Vfr. The pyoverdine regulation through Gac system is independent of the well-known Fur repressor since no differences were observed for the level of Fur protein between wild-type strain, the *gac* mutants, and the Rsm-overexpressing strains in any condition tested. These results suggest that other unknown repressors are the responsible genes in Gac-mediated repression of motility and in pyoverdine synthesis in *P. fluorescens* F113.

INTRODUCTION

The Gac system conforms a conserved (de Souza *et al.*, 2003) global regulatory system that regulates the production of the majority of exoproducts and virulence factors in the pseudomonads, independently of their life-style (Heeb & Haas, 2001).

In the opportunistic pathogen *Pseudomonas aeruginosa*, the Gac system positively regulates the production of the autoinducer N-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, lipase (Reimmann *et al.*, 1997) and elastase production (Burrowes *et al.*, 2005), being necessary for full virulence in animal and plant hosts (Rahme *et al.*, 1995). The Gac system also regulates most of the virulence factors that have been identified in the insect pathogen *P. entomophila* (Vodovar *et al.*, 2006). In phytopathogenic pseudomonads, such as *P. syringae*, the Gac system has been implicated in lesion formation and the production of protease and the phytotoxin syringomycin (Rich *et al.*, 1994), swarming motility (Kinscherf & Willis, 1999) and alginate production (Willis *et al.*, 2001), acting as a master regulator (Chatterjee *et al.*, 2003). In saprophytic pseudomonads such as *P. fluorescens, P. putida, P. aeurofaciens,* and others, the Gac system has been shown to regulate the production of secondary metabolites such as the fungicide 2,4-diacetylphloroglucinol (DAPG), cyanide, pyoluteorin, phenazine, and the phytohormone indole-3-acetic acid (Aarons *et al.*, 2000; Kang *et al.*, 2006; Laville *et al.*, 1992; Zhang & Pierson, 2001), extracellular enzymes and fluorescent siderophores (Liao *et al.*, 1996; Sacherer *et al.*, 1994), and lipopeptides such as amphisin (Koch *et al.*, 2002) and putisolvin (Dubern *et al.*, 2005). Mutations in the Gac system may result in the loss of biocontrol ability (Laville *et al.*, 1992).

The Gac system acts, in the regulation of the production of most of these exoproducts, as an activator. This system, in response to a yet unidentified signal

produced during the transition to stationary phase (Zuber *et al.*, 2003), activates the transcription of several small regulatory RNAs termed *rsmX*, *rsmY* and *rsmZ*. Different *Pseudomonas* produce one, two or three of these RNAs (Aarons *et al.*, 2000; Kay *et al.*, 2005; Kay *et al.*, 2006). In turn, the small RNAs titrate two RNA-binding proteins RsmA and RsmE that in the absence of the small RNAs bind to the 5´ regions of target messenger RNAs repressing their translation (Reimmann *et al.*, 2005). However, in a few cases, negative regulation by the Gac system has been observed. This is the case for rhamnolipids and lipase production, and swarming motility in *P. aeruginosa* PAO1 (Heurlier *et al.*, 2004).

We have previously shown that two traits of *Pseudomonas fluorescens* F113 which are important for rhizosphere colonization and biocontrol ability, swimming motility and pyoverdine production, are also under negative control by the Gac system, since mutants affected in either of the *gac* genes produce larger swimming haloes than the wild-type strain and produce the siderophore pyoverdine even under high iron growth conditions (Martínez-Granero *et al.*, 2005; Martínez-Granero *et al.*, 2006).

Many bacteria produce iron chelators, called siderophores, which make iron available to the cell. Fluorescent pseudomonads can produce several different siderophores such as pyoverdine, pyochelin, etc (Cox *et al.*, 1981; Mercado-Blanco *et al.*, 2001; Meyer, 2000; Mossialos *et al.*, 2000). The production of the major siderophore pyoverdine and its cognate receptor is effected by a regulation cascade involving a general repressor, Fur, and its co-repressor ferrous iron, which control the transcription of extracytoplasmic sigma factors PvdS and FpvI (Redly & Poole, 2003; Visca *et al.*, 2002). In turn, PvdS is needed for the transcription of several pyoverdine biosynthesis genes (Leoni *et al.*, 2000; Wilson *et al.*, 2001), and FpvI for its cognate receptor (Redly & Poole, 2003).

The aim of this work was to investigate the mechanism of negative regulation of swimming motility and pyoverdine production by the Gac system. The obtained results show that negative regulation also occurs through the Rsm pathway. In the absence of an active Gac system, it apparently prevents the translation of yet unidentified repressors. In the case of swimming motility, the lack of the Gac system results in the overexpression of the *fleQ* gene, encoding the major transcriptional activator of flagellar filament synthesis and in enhanced motility.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions

All the *Pseudomonas fluorescens* strains used here are derivatives of the biocontrol strain F113 (Shanahan *et al.*, 1992), and Pf0-1 (Compeau *et al.*, 1988).

All the PCR fragments obtained in this study were cloned in the pGEM-T Easy vector (Promega). F113 *vfr* mutant was obtained by single homologous recombination of amplified internal fragment from *vfr* gene cloned into the suicide vector pVIK107 (Kalogeraki & Winans, 1997). Overexpression of *rsmA* and *rsmE* genes was achieved by cloning them under the control of the strong *nptII* promoter present in the pFAJ1709 plasmid (Dombrecht *et al.*, 2001). Plasmids were mobilized into *P. fluorescens* by triparental matings, using pRK2013 as the helper plasmid (Figurski & Helinski, 1979).

P. fluorescens strains were grown in SA medium (Scher & Baker, 1982) overnight at 28°C; solid growth medium contained 1.5% (w/v) purified agar. *Escherichia coli* strains were grown overnight in Luria-Bertani (LB) medium (Bertani, 1951) at 37°C.

The following antibiotics were used, when required, at the indicated concentrations: rifampicin, 100 μg/ml; ampiciline, 100 μg/ml; tetracycline, 10 μg/ml for *E. coli* or 70 μg/ml for *P. fluorescens*; and kanamycin, 25 μg/ml for *E. coli* or 50 μg/ml for *P. fluorescens*.

Pyoverdine production was observed under UV light on LB plates supplemented with tetracycline in order to maintain plasmids.

Transmission electron microscopy

Formvar-coated grids were placed on the top of a drop of bacterial cells for 30 s to allow bacterial adhesion. Grids were stained for 1 min with a 1 % solution of potassium phosphotungstate and washed for 1 min with a drop of water.

DNA techniques

Standard methods were used for DNA extraction, gene cloning, plasmid preparations and agarose gel electrophoresis (Sambrook *et al.*, 1989). Southern blottings were performed with a non-radioactive detection kit, and a chemiluminescence method was used to detect hybridization signals according to the instructions of the manufacturer (Roche Boehringer Mannheim). PCR reactions were performed using the *Tth* enzyme (Biotools) under standard conditions. Primer sequences are available on request. DNA sequencing was done by chain-termination method using DyeDeoxy terminator cycle sequencing kit protocol as described by the manufacturer (Applied Biosystem). Sequence analysis was performed with software from the Genetics Computer Group (Madison, WI, USA) and the BLAST programs.

Swimming assays

SA medium plates containing 0.3% purified agar were used to test swimming abilities. Complementation assays were done using the plasmid-cloned *gacA* gene from *P. fluorescens* CHA0 (Laville *et al.*, 1992) and the *gacS* gene from *P. syringae* pv. *syringae* B728a (Hrabak & Willis, 1992), the empty pFAJ1709 plasmid was used as a control. The cells from exponentially growing cultures were inoculated into the plate using a toothpick. Swimming haloes were measured after 18, 24, and 42 h of inoculation. Every assay was performed three times with three replicates each time.

Protein extraction and Western blots

Proteins were extracted from 200 ml exponential ($O.D._{600}$ = 0.3) and stationary ($O.D._{600}$ = 3.5) phase grown cultures. In order to detach the flagella, the cultures were agitated by vortexing for 2 min and then centrifuged for 20 min at 12000 r.p.m. Total proteins were extracted from the pellet with Laemmli buffer (Laemmli, 1970) and extracellular proteins were extracted from the supernatant by precipitation for 16 h at 4ºC with 10% (w/v) trichloroacetic acid, followed by two washes with chilled acetone, and were finally resuspended in Laemmli buffer. Proteins were electrophoresed in 12% acrylamide gels and stained with Coomassie blue. The same electrophoretic conditions were used for Western blotting. Acrylamide gels were transferred to nitrocellulose membranes for 1 h under standard conditions. The membranes were incubated with a 1:10000 dilution of an anti-flagellin antiserum (Dekkers *et al.*, 1998) or a 1:100 dilution of an anti-Fur antiserum (Ochsner & Vasil, 1996) for 1 h and then with a peroxidase-tagged secondary antibody (anti-rabbit immunoglobulin) for 1 h. The enhanced chemiluminescence (ECL) method and Hyperfilm ECL (Amersham Biosciences) were used for development.

Gene expression analysis

Translational *lacZ* fusion of the *vfr* gene was constructed by directionally cloning an internal fragment from *vfr* gene into the pVIK107 suicide vector (Kalogeraki & Winans, 1997). The construct was integrated into the F113 genome by triparental mating, and single homologous recombinants were checked by Southern blot. β-Galactosidase activity was determined according to Miller (1972).

Total RNA was extracted according to the instructions of the manufacturer (Roche Boehringer Manheim) from *P. fluorescens* strains grown at 28ºC in LB medium. The concentration of RNA was spectrophotometrically determined and normalized by using 16S RNA as internal control. *fleQ*, *fliC*, and *pvdA* expression was measured into different backgrounds by using One-Step RT-PCR kit from Amersham Biosciences and iScript One-Step RT-PCR kit with SYBR Green from Bio-Rad.

RESULTS

The Gac system regulates motility and pyoverdine production through the Rsm pathway

Besides the transcriptional activation of the genes encoding the small RNAs *rsmX, Y* and *Z*, positive regulation by the Gac system of other traits occurs at a posttranscriptional level through RsmA and RsmE, two apparently redundant RNA-binding proteins (Reimmann *et al.*, 2005). To test whether negative regulation of motility and pyoverdine production also occurred through this pathway, we hypothesized that in this case the overproduction of either of the Rsm proteins would mimic the phenotype of a *gac* mutant. In order to overexpress the *rsmA* and *rsmE* genes, we designed primers from their 5´ and 3´ regions based in the sequences of these genes from other pseudomonads´ genomes. The primers were

used to amplify both *rsm* genes from *P. fluorescens* F113 genomic DNA. The resulting products from the PCR reactions were cloned in the pGEM-T Easy vector and fully sequenced. Sequencing of the PCR products confirmed that they corresponded to the *rsmA* and *rsmE* genes from strain F113 since they showed high homology (93% for *rsmA* gene, and 89% for *rsmE* gene at nucleotide level and 100% for both genes at aminoacid level) with their counterparts in other pseudomonads.

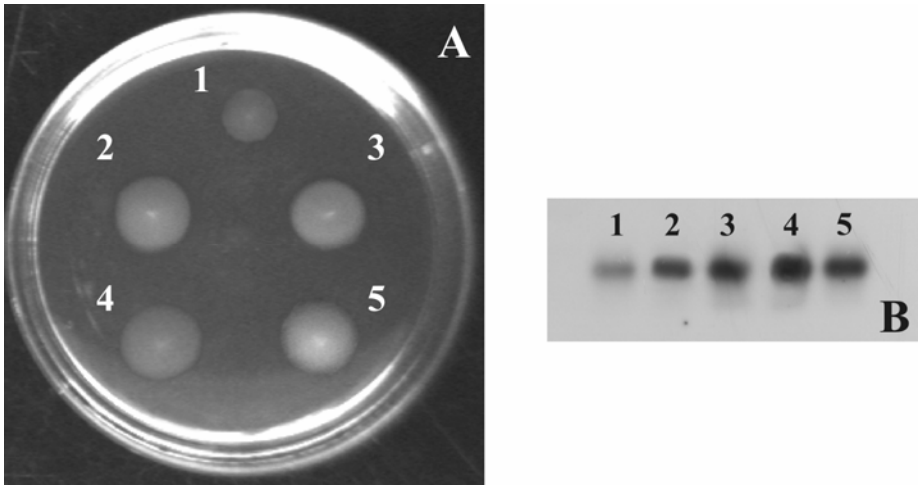

Figure 5.1. Analysis of the swimming motility (A) and *pvdA* expression (B) of *P. fluorescens* F113 wild-type (1), F113 *gacA* mutant (2), F113 *gacS* mutant (3), F113 *rsmA*+ (4), and F113 *rsmE*+ (5).

The DNA fragments containing each of the *rsm* genes were independently subcloned in the expression vector pFAJ1709 under the control of the *nptII* promoter (Dombrecht *et al.*, 2001) and introduced into *P. fluorescens* F113 by triparental mating, to generate strains F113 *rsmA*+ and F113 *rsmE*+. As shown in Fig. 5.1, while the wild-type strain F113 showed normal motility and low levels of *pvdA* gene expression (L-ornithine N^5-oxygenase involved in pyoverdine biosynthesis) on LB medium, overexpression of either of the *rsm* genes in F113

resulted in motility and *pvdA* gene expression phenotypes identical to the *gacA* and *gacS* mutants. These results clearly showed that in *P. fluorescens* F113, the Gac two-component system negatively regulates motility and pyoverdine production through the RNA-binding proteins RsmA and RsmE, that is, through the same pathway that positive regulation occurs.

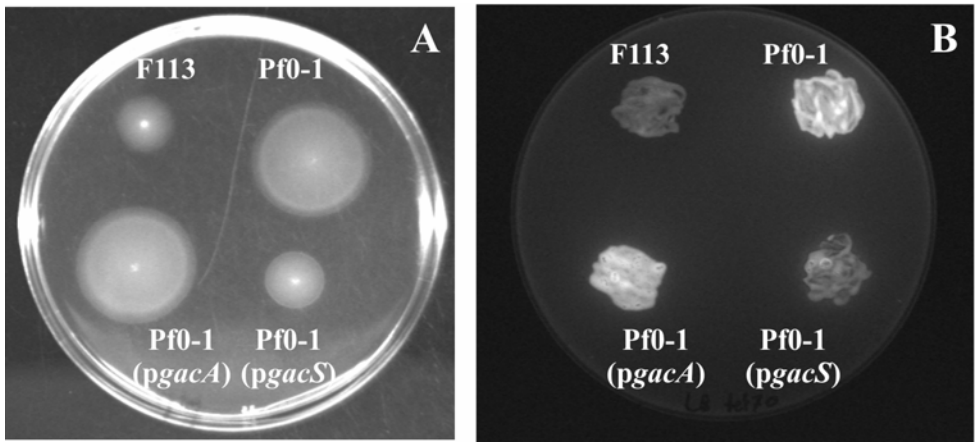

Figure 5.2. Analysis of the swimming motility (A) and pyoverdine production (B) of *P. fluorescens* F113, and *P. fluorescens* Pf0-1. Complementation analysis was performed with *P. fluorescens* Pf0-1 strain containing the cloned *gac* genes (p*gacA*/*S*). The empty vectors (not shown) had no effect on halo formation and pyoverdine production.

In order to extend this observation to other *P. fluorescens* strains, we tested the motility and pyoverdine production phenotype in *P. fluorescens* Pf0-1, a model strain whose genome has been totally sequenced. Surprisingly, the motility and pyoverdine production phenotype of this strain resembled the phenotype of *gac* mutants in strain F113 (Fig. 5.2). However, when the cloned *gacS* from *P. syringae* (Hrabak & Willis, 1992), that complements the *gacS* mutant of strain F113, was introduced in Pf0-1 the motility and pyoverdine production phenotype was similar to the wild-type F113 strain (Fig. 5.2). The cloned *gacA* (Laville *et al.*, 1992) had

no effect in the phenotype of Pf0-1. These results indicate that the Pf0-1 strain that we tested harbours a mutation in the *gacS* gene, and more importantly, that in *P. fluorescens* Pf0-1 motility and pyoverdine production are also under the negative control of the Gac system.

Negative regulation of motility by the Gac system acts through downregulation of the *fleQ* gene transcription

The *fleQ* gene encodes the major regulator of flagellar biosynthesis (Capdevila *et al.*, 2004; Dasgupta *et al.*, 2003). We have previously shown that hypermotile phenotypic variants of *P. fluorescens* F113 were characterized by overproduction of flagellin (FliC) and longer flagella (Sánchez-Contreras *et al.*, 2002). As these phenotypes could be controlled by the FleQ protein, we tested the expression of the *fleQ* and *fliC* genes in different backgrounds. Fig 5.3 shows the RT-PCR products obtained from total RNA of the different strains. The expression of the *fleQ* gene was higher in the *gacA* and *gacS* mutants than in the wild-type strain. These results were confirmed by real time RT-PCR that showed that in the *gac* mutants the expression of *fleQ* was between 18 and 22 times higher than in the wild-type strain. The expression of *fleQ* was also higher in the strains that overexpressed the *rsmA* and *rsmE* genes (Fig. 5.3). On the other hand, the expression of the FleQ-regulated *fliC* gene showed the same pattern that the *fleQ* gene, with higher expression in the *gac* mutants and the strains overexpressing the *rsm* genes than in the wild-type strain. These results clearly show that the negative regulation of motility by the Gac system acts on the flagellar filament synthesis through its major regulator FleQ.

Since the Gac system regulates secondary metabolism, especially at the transition from exponential to stationary growth, we hypothesized that the role of the Gac system on motility could be to downregulate flagellar synthesis during exponential growth. To test this hypothesis, total proteins from the wild-type strain,

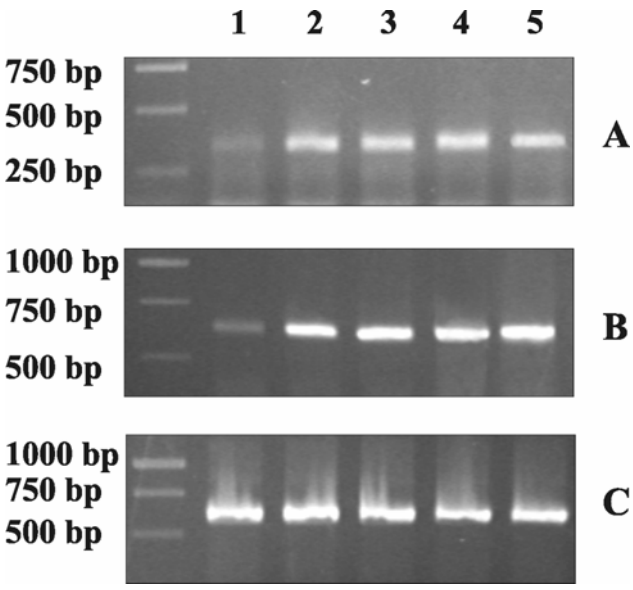

Figure 5.3. RT-PCR of *fleQ* gene (A), *fliC* gene (B), and *16S* gene (C) of *P. fluorescens* F113 wild-type (1), F113 *gacA* mutant (2), F113 *gacS* mutant (3), F113 *rsmA*+ (4), and F113 *rsmE*+ (5) during exponential phase ($O.D._{600}$ = 0.3).

both *gac* mutants and the strains overexpressing the *rsm* genes were precipitated from the growth medium during exponential phase ($O.D._{600}$ = 0.3) and stationary phase ($O.D._{600}$ = 3.5). These proteins were probed with an anti-FliC (flagellin) antiserum. As showed in Fig. 5.4, during exponential phase the *gac* mutants and the strains overexpressing either of the *rsm* genes produced a higher amount of flagellin than the wild-type strain. However, during stationary phase no differences in flagellin production were observed with the wild-type strain. Furthermore, transmission electron microscopy of negatively stained samples from the *gac* mutants and the wild-type strain showed that the percentage of flagellated cells were higher in the *gac* mutants than in the wild-type strain during exponential growth (7.69% for wild-type strain, and 37.37% for *gac* mutants) but not during stationary phase (76.97% for wild-type strain, and 84.35% for *gac* mutants). These

results support the hypothesis of the role of the Gac system limiting flagella biosynthesis during exponential growth phase.

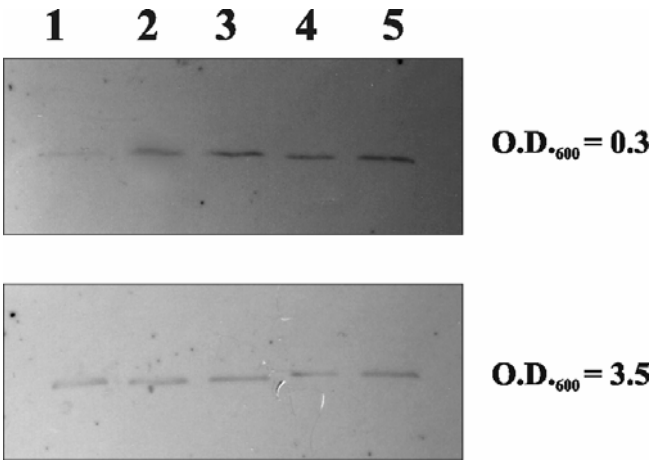

Figure 5.4. Western blot analysis of external proteins from *P. fluorescens* F113 wild-type (1), F113 *gacA* mutant (2), F113 *gacS* mutant (3), F113 *rsmA*+ (4), and F113 *rsmE*+ (5) during exponential phase ($O.D._{600}$ = 0.3), and stationary phase ($O.D._{600}$ = 3.5), reacted with an anti-flagellin antiserum. The observed band is approximately 35 KDa and corresponds to FliC.

Gac-mediated downregulation of *fleQ* expression is independent of Vfr

Gac regulation through the Rsm pathway takes place at translational level since the RsmA and E proteins bind specific messenger RNAs blocking their translation (Blumer *et al.*, 1999; Reimmann *et al.*, 2005). For negative regulation of motility, the RNA blocked should encode a repressor of *fleQ* transcription. Although several proteins such as MorA, FleN and AlgT have been shown to modulate *fleQ* expression in different pseudomonads (Choy *et al.*, 2004; Dasgupta & Ramphal, 2001; Tart *et al.*, 2005), a direct role in repressing *fleQ* transcription by binding to the promoter region has only been shown for the global regulatory

protein Vfr in *P. aeruginosa* (Dasgupta *et al.*, 2002). Furthermore, Vfr has been implicated in the regulation of two Gac-controlled traits in *P. aeruginosa*: elastase and pyocyanin production (Beatson *et al.*, 2002).

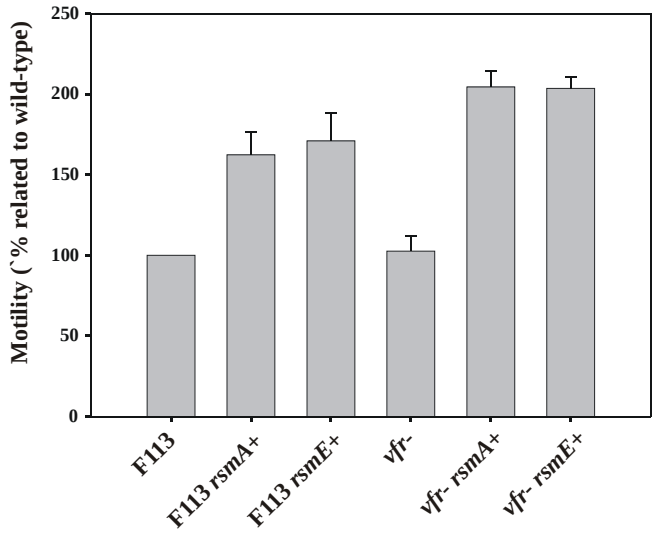

Figure 5.5. Swimming motility of *P. fluorescens* F113, its *vfr* isogenic mutant (*vfr*-), and derivatives overexpressing *rsm* genes (*rsmA*+, and *rsmE*+). Arithmetic means and standard deviations are presented.

Since the F113 *fleQ* promoter region contains a putative Vfr binding site, we decided to test whether Vfr was implicated in Gac-mediated *fleQ* downregulation. For this purpose, we amplified an internal fragment of *vfr* gene from F113 genomic DNA and ligated it to the *lacZ* gene present in pVIK107 creating a translational fusion. This fusion was integrated into the F113 genome by homologous recombination, and the resulting strain F113 Vfr::*lacZ* was checked by Southern-blot, using the amplified *vfr* fragment as the probe. If Vfr was implicated in Gac-mediated regulation we would expect that when *rsmA* or *rsmE* were overexpressed, the RsmA and RsmE proteins would bind to the *vfr* messenger RNA

blocking its translation. However, β-galactosidase activity of the *vfr*::*lacZ* translational fusion was similar in the strains overexpressing either of the *rsm* genes (39.64±1.72 Miller units for *rsmA*+, and 36.26±0.73 Miller units for *rsmE*+) and in the wild-type (37.18±3.99 Miller units), indicating that Gac-dependent downregulation of *fleQ* is independent of Vfr. Furthermore, the introduction of the *vfr*::*lacZ* fusion in F113 generated a *vfr* mutant, as checked by Southern-blot. As shown in Fig. 5.5, this mutant did not show any difference in motility with the wild-type strain. The overexpression of either of the *rsm* genes in the mutant resulted in an increase in motility (Fig. 5.5), clearly showing that the effect of RsmA and RsmE in motility was independent of the presence of a functional *vfr* gene. It is likely that a yet unidentified transcriptional repressor of *fleQ* is the target of the Rsm proteins, and the final responsible of the low flagellar biosynthesis during the exponential phase.

Gac-mediated repression of pyoverdine production is independent of Fur

We have observed that besides production of pyoverdine under iron sufficient conditions, *gac* mutants produce pyoverdine under iron limiting conditions earlier in growth phase than the wild-type strain. When growing on SA medium production of the green coloured pyoverdine is evident at an optical density of 1.25, corresponding with early stationary phase. Conversely, the *gacA* and *gacS* mutants, as well as the strains F113 *rsmA*+ and F113 *rsmE*+ start production of pyoverdine at an optical density of 0.8, which correspond to the end of the exponential phase. This observation suggests that similarly to motility, a physiological role of the Gac system is to repress pyoverdine production during exponential growth

.

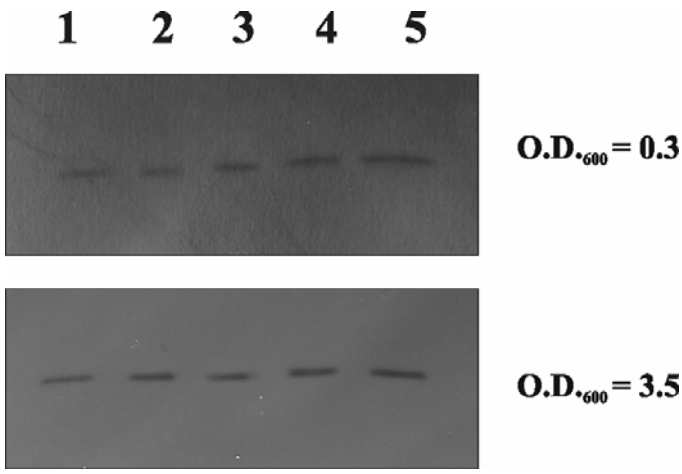

Figure 5.6. Western blot analysis of total proteins from *P. fluorescens* F113 wild-type (1), F113 *gacA* mutant (2), F113 *gacS* mutant (3), F113 *rsmA*+ (4), and F113 *rsmE*+ (5) during exponential phase ($O.D._{600}$ = 0.3), and stationary phase ($O.D._{600}$ = 3.5), reacted with an anti-Fur antiserum.

It is well known that under iron sufficiency, pyoverdine production is tightly repressed by the Fur repressor. Therefore the *fur* mRNA is a potential target for the RsmA and RsmE proteins. To test this hypothesis we used an anti-Fur antiserum to probe soluble proteins from the different strains, both during exponential and stationary phase. Fig 5.6 shows that no differences were observed for the level of Fur protein at any growth phase between the wild-type strain, the *gac* mutants and the strains overexpressing the *rsm* genes, indicating that Gac repression of pyoverdine production is independent of Fur.

DISCUSSION

The GacA/GacS two component system (Gac system) is a major regulator of secondary metabolism in many Gram-negative bacteria including pseudomonads (Heeb & Haas, 2001). Traits regulated by this system in several species of

Pseudomonas include the production of extracellular metabolites such as exoprotease, hydrogen cyanide, pyocyanin and elastase that are important for virulence and/or biocontrol applications (Aarons *et al.*, 2000; Burrowes *et al.*, 2005; Haas & Keel, 2003; Heeb & Haas, 2001; Laville *et al.*, 1992; Rahme *et al.*, 1995; Reimmann *et al.*, 1997). Although for most traits the Gac system acts as a positive regulator, for some traits such as swarming motility, rhamnolipids and lipase production it may function as a negative regulator (Heurlier *et al.*, 2004). This negative role of the Gac system is especially clear for two traits in *P. fluorescens* F113: swimming motility and production of the siderophore pyoverdine (Martínez-Granero *et al.*, 2005; Martínez-Granero *et al.*, 2006). Mutations in the *gacA* or *gacS* genes results in increased motility and in the production of pyoverdine even under high iron growth conditions. The relevance of these traits and of the Gac system for rhizosphere colonization is highlighted by the fact that phenotypic variants arising during rhizosphere colonization harbour mutations in the *gac* genes, being more motile than the wild-type strain. Furthermore, several of these variants, selected because of increased motility, were more competitive for rhizosphere colonization than the wild-type strain (Martínez-Granero *et al.*, 2006). Here, we have shown that the role of the Gac system in repressing swimming motility and pyoverdine production can be extended to other strain of *P. fluorescens* (Fig. 5.2). This and the recent finding that in *P. aeruginosa* the FliC and FliD proteins are the most highly overproduced proteins in *gac* mutants (Kay *et al.*, 2006), suggest that negative regulation by the Gac system of these traits may be a general feature in pseudomonads.

Activation through the Gac system occurs post-transcriptionally. Briefly, an unidentified bacterial signal stimulates autophosphorylation of the GacS sensor (Zuber *et al.*, 2003). The phosphate group is then transferred to the response regulator GacA by a phospho-relay mechanism, activating directly or indirectly the transcription of genes encoding small RNAs, termed *rsmX*, *Y* and *Z*. These riboregulators bind to two RNA-binding proteins, RsmA and E, that have the ability

to bind specific mRNAs blocking their transcription (Valverde *et al.*, 2004). In such system, an active Gac system results in the RsmA and E proteins bound to the small regulatory RNAs and therefore the RsmA and E target mRNAs are translated. Conversely, in the absence of a functional Gac system (for instance strains harbouring a *gac* mutation), the RsmA and E proteins would be bound to their target mRNAs that would not be transcribed. This model easily explains positive regulation, since translation of the target mRNAs is required for the production of the trait. Here we show that the Rsm pathway is also used for negative regulation in *P. fluorescens*, since overexpression of either of the *rsmA* or *E* genes mimic the phenotypes of the *gac* mutants. Our results also show that for repression of swimming motility and pyoverdine production the RsmA and RsmE proteins are functionally equivalent (Fig. 5.1). This functional equivalence has also been shown for other positively regulated traits such as exoprotease, hydrogen cyanide, and 2,4-diacetylphloroglucinol in *P. fluorescens* CHAO (Reimmann *et al.*, 2005). However, it is not known whether it is true for all Rsm-controlled traits. It is interesting to note that several pseudomonads, such as *P. aeruginosa*, produce a single Rsm protein (Pessi *et al.*, 2001).

We have previously shown that hypermotile phenotypic variants of strain F113 isolated from the rhizosphere harboured *gac* mutations, produced higher amounts of the FliC protein and possess longer flagella than the wild-type strain (Sánchez-Contreras *et al.*, 2002). Since the major activator of flagella synthesis is the FleQ protein (Dasgupta *et al.*, 2003), we decided to test whether the Gac system acted through the *fleQ* gene to regulate swimming motility. Our results clearly show that the Gac system dramatically influences the level of transcription of the *fleQ* and *fliC* genes and that this influence is enforced through the RsmA and RsmE proteins (Fig. 5.3). These results are consistent with those recently reported in *P. aeruginosa* that show that in a *gacA* and a *rsmYZ* mutants, FliC and FliD (the flagellar cap protein) had increased expression (between 7.5 and 10.2-fold) when compared to

wild-type strain, being the most overproduced proteins in both mutants (Kay *et al.*, 2006).

Since Gac regulation of both motility and pyoverdine production occurs through the Rsm pathway, a direct effect on the transcription of activators such as *fleQ* or *pvdS* can be discarded. Two alternative ways are possible. The RsmA and E proteins could bind to the mRNA of the transcriptional activators stabilizing them or the Rsm proteins would bind to the mRNAs encoding transcriptional repressors of the activator genes. The former possibility has been shown to occur with the RsmA homologue CsrA in *Escherichia coli* (Wei et al., 2001). In this bacterium, CsrA binds the mRNA of the *flhDC* genes, which encode the master operon regulating flagellar biosynthesis. However, this model is not consistent with the high increase (ca. twenty fold) that we have observed in the levels of *fleQ* mRNA in F113 *gac* mutants compared to the wild-type strain. The second possibility, i.e. the RsmA and E binding of mRNAs encoding transcriptional repressors could explain the observed phenotypes of *gac* mutants. We have tested the levels of Vfr and Fur, known transcriptional repressors of the *fleQ* gene and *pvdS* gene, respectively (Dasgupta *et al.*, 2002; Visca *et al.*, 2002). Our results (Figs. 5.4-5.6) discard the implication of neither of these proteins in Gac regulation of swimming motility and pyoverdine production, suggesting that yet unknown transcriptional repressors are the responsible genes in Gac-mediated repression.

Gac-mediated positive regulation typically occurs in the transition from exponential to stationary phase. In this sense, the Gac system has been defined as a global activator of secondary metabolism in stationary phase. Furthermore, a relation between the Gac system and RpoS, the stationary phase sigma factor, has been described (Chatterjee *et al.*, 2003; Heeb *et al.*, 2005; Whistler *et al.*, 1998). Here, we present evidence showing that in *P. fluorescens* the Gac system is also active during exponential phase, being able to repress flagellar synthesis and pyoverdine production at this stage. Therefore, we propose a second physiological

role for the Gac system: the repression or downregulation of specific traits during exponential phase.

ACKNOWLEDGEMENTS

We are grateful to Ine Mulders and Ben Lugtenberg for the anti-flagellin antiserum, Mike Vasil for anti-Fur antiserum, and Stuart Levy for *P. fluorescens* Pf0-1 strain. This work was funded by the Spanish Ministry of Science and Education grant BIO2003-03412 and Comunidad de Madrid grant GR/AMB/0084/2004 and by the Research Program MICROAMBIENTE-CM from Comunidad de Madrid. M. Martin was the recipient of a Ramón y Cajal contract from the Spanish Ministry of Science and Education.

REFERENCES

Aarons, S., Abbas, A., Adams, C., Fenton, A. & O'Gara, F. (2000). A regulatory RNA (PrrB RNA) modulates expression of secondary metabolite genes in *Pseudomonas fluorescens* F113. *J Bacteriol* **182**, 3913-3919.

Beatson, S. A., Whitchurch, C. B., Sargent, J. L., Levesque, R. C. & Mattick, J. S. (2002). Differential regulation of twitching motility and elastase production by Vfr in *Pseudomonas aeruginosa*. *J Bacteriol* **184**, 3605-3613.

Bertani, G. (1951). Studies on Lysogenesis. 1. The Mode of Phage Liberation by Lysogenic *Escherichia coli*. *J Bacteriol* **62**, 293-300.

Blumer, C., Heeb, S., Pessi, G. & Haas, D. (1999). Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. *Proc Natl Acad Sci U S A* **96**, 14073-14078.

Burrowes, E., Abbas, A., O'Neill, A., Adams, C. & O'Gara, F. (2005). Characterisation of the regulatory RNA *rsmB* from *Pseudomonas aeruginosa* PAO1. *Res Microbiol* **156**, 7-16.

Capdevila, S., Martínez-Granero, F. M., Sánchez-Contreras, M., Rivilla, R. & Martín, M. (2004). Analysis of *Pseudomonas fluorescens* F113 genes implicated in flagellar filament synthesis and their role in competitive root colonization. *Microbiol* **150**, 3889-3897.

Compeau, G., Alachi, B. J., Platsouka, E. & Levy, S. B. (1988). Survival of rifampin-resistant mutants of *Pseudomonas fluorescens* and *Pseudomonas putida* in soil systems. *Appl Environ Microbiol* **54**, 2432-2438.

Cox, C. D., Rinehart, K. L., Moore, M. L. & Cook, J. C. (1981). Pyochelin: novel structure of an iron chelating growth promoter for *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **78**, 4256-4260.

Chatterjee, A., Cui, Y. Y., Yang, H. L., Collmer, A., Alfano, J. R. & Chatterjee, A. K. (2003). GacA, the response regulator of a two-component system, acts as a master regulator in *Pseudomonas syringae* pv. *tomato* DC3000 by controlling regulatory RNA, transcriptional activators, and alternate sigma factors. *Mol Plant-Microbe Interact* **16**, 1106-1117.

Choy, W. K., Zhou, L., Syn, C. K. C., Zhang, L. H. & Swarup, S. (2004). MorA defines a new class of regulators affecting flagellar development and biofilm formation in diverse *Pseudomonas* species. *J Bacteriol* **186**, 7221-7228.

Dasgupta, N. & Ramphal, R. (2001). Interaction of the antiactivator FleN with the transcriptional activator FleQ regulates flagellar number in *Pseudomonas aeruginosa*. *J Bacteriol* **183**, 6636-6644.

Dasgupta, N., Ferrell, E. P., Kanack, K. J., West, S. E. H. & Ramphal, R. (2002). *fleQ*, the gene encoding the major flagellar regulator of *Pseudomonas aeruginosa*, is σ^{70} dependent and is downregulated by Vfr, a homolog of *Escherichia coli* cyclic AMP receptor protein. *J Bacteriol* **184**, 5240-5250.

Dasgupta, N., Wolfgang, M. C., Goodman, A. L., Arora, S. K., Jyot, J., Lory, S. & Ramphal, R. (2003). A four-tiered transcriptional regulatory circuit controls flagellar biogenesis in *Pseudomonas aeruginosa*. *Mol Microbiol* **50**, 809-824.

de Souza, J. T., Mazzola, M. & Raaijmakers, J. M. (2003). Conservation of the response regulator gene *gacA* in *Pseudomonas* species. *Environ Microbiol* **5**, 1328-1340.

Dekkers, L. C., Phoelich, C. C., van der Fits, L. & Lugtenberg, B. J. J. (1998). A site-specific recombinase is required for competitive root colonization by *Pseudomonas fluorescens* WCS365. *Proc Natl Acad Sci U S A* **95**, 7051-7056.

Dombrecht, B., Vanderleyden, J. & Michiels, J. (2001). Stable RK2-derived cloning vectors for the analysis of gene expression and gene function in gram-negative bacteria. *Mol Plant-Microbe Interact* **14**, 426-430.

Dubern, J. F., Lagendijk, E. L., Lugtenberg, B. J. J. & Bloemberg, G. V. (2005). The heat shock genes *dnaK*, *dnaJ*, and *grpE* are involved in regulation of putisolvin biosynthesis in *Pseudomonas putida* PCL1445. *J Bacteriol* **187**, 5967-5976.

Figurski, D. H. & Helinski, D. R. (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci U S A* **76**, 1648-1652.

Haas, D. & Keel, C. (2003). Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. *Annu Rev Phytopathol* **41**, 117-153.

Heeb, S. & Haas, D. (2001). Regulatory roles of the GacS/GacA two-component system in plant-associated and other Gram-negative bacteria. *Mol Plant-Microbe Interact* **14**, 1351-1363.

Heeb, S., Valverde, C., Gigot-Bonnefoy, C. & Haas, D. (2005). Role of the stress sigma factor RpoS in GacA/RsmA-controlled secondary metabolism and resistance to oxidative stress in *Pseudomonas fluorescens* CHA0. *FEMS Microbiol Lett* **243**, 251-258.

Heurlier, K., Williams, F., Heeb, S., Dormond, C., Pessi, G., Singer, D., Camara, M., Williams, P. & Haas, D. (2004). Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/*rsmZ* system in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **186**, 2936-2945.

Hrabak, E. M. & Willis, D. K. (1992). The *lemA* gene required for pathogenicity of *Pseudomonas syringae* pv. *syringae* on bean is a member of a family of two-component regulators. *J Bacteriol* **174**, 3011-3020.

Kalogeraki, V. S. & Winans, S. C. (1997). Suicide plasmids containing promoterless reporter genes can simultaneously disrupt and create fusions to target genes of diverse bacteria. *Gene* **188**, 69-75.

Kang, B. R., Yang, K. Y., Cho, B. H., Han, T. H., Kim, I. S., Lee, M. C., Anderson, A. J. & Kim, Y. C. (2006). Production of indole-3-acetic acid in the plant-beneficial strain *Pseudomonas chlororaphis* O6 is negatively regulated by the global sensor kinase GacS. *Curr Microbiol* **52**, 473-476.

Kay, E., Dubuis, C. & Haas, D. (2005). Three small RNAs jointly ensure secondary metabolism and biocontrol in *Pseudomonas fluorescens* CHA0. *Proc Natl Acad Sci U S A* **102**, 17136-17141.

Kay, E., Humair, B., Dénervaud, V., Riedel, K., Spahr, S., Eberl, L., Valverde, C. & Haas, D. (2006). Two GacA-dependent small RNAs modulate the quorum-sensing response in *Pseudomonas aeruginosa*. *J Bacteriol* **188**, 6026-6033.

Kinscherf, T. G. & Willis, D. K. (1999). Swarming by *Pseudomonas syringae* B728a requires *gacS* (*lemA*) and *gacA* but not the acyl-homoserine lactone biosynthetic gene *ahlI*. *J Bacteriol* **181**, 4133-4136.

Koch, B., Nielsen, T. H., Sorensen, D., Andersen, J. B., Christophersen, C., Molin, S., Givskov, M., Sorensen, J. & Nybroe, O. (2002). Lipopeptide production in *Pseudomonas* sp. strain DSS73 is regulated by components of sugar beet seed exudate via the Gac two-component regulatory system. *Appl Environ Microbiol* **68**, 4509-4516.

Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of head of bacteriophage T4. *Nature* **227**, 680-685.

Laville, J., Voisard, C., Keel, C., Maurhofer, M., Defago, G. & Haas, D. (1992). Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root-rot of tobacco. *Proc Natl Acad Sci U S A* **89**, 1562-1566.

Leoni, L., Orsi, N., de Lorenzo, V. & Visca, P. (2000). Functional analysis of PvdS, an iron starvation sigma factor of *Pseudomonas aeruginosa*. *J Bacteriol* **182**, 1481-1491.

Liao, C. H., McCallus, D. E., Wells, J. M., Tzean, S. S. & Kang, G. Y. (1996). The *repB* gene required for production of extracellular enzymes and fluorescent siderophores in *Pseudomonas viridiflava* is an analog of the *gacA* gene of *Pseudomonas syringae*. *Can J Microbiol* **42**, 177-182.

Martínez-Granero, F., Capdevila, S., Sánchez-Contreras, M., Martín, M. & Rivilla, R. (2005). Two site-specific recombinases are implicated in phenotypic variation and competitive rhizosphere colonization in *Pseudomonas fluorescens*. *Microbiol* **151**, 975-983.

Martínez-Granero, F., Rivilla, R. & Martin, M. (2006). Rhizosphere selection of highly motile phenotypic variants of *Pseudomonas fluorescens* with enhanced competitive colonization ability. *Appl Environ Microbiol* **72**, 3429-3434.

Mercado-Blanco, J., van der Drift, K. M. G. M., Olsson, P. E., Thomas-Oates, J. E., van Loon, L. C. & Bakker, P. A. H. M. (2001). Analysis of the *pmsCEAB* gene cluster involved in biosynthesis of salicylic acid and the siderophore pseudomonine in the biocontrol strain *Pseudomonas fluorescens* WCS374. *J Bacteriol* **183**, 1909-1920.

Meyer, J. M. (2000). Pyoverdines: pigments, siderophores and potential taxonomic markers of fluorescent *Pseudomonas* species. *Arch Microbiol* **174**, 135-142.

Miller, J. H. (1972). Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Mossialos, D., Meyer, J. M., Budzikiewicz, H., Wolff, U., Koedam, N., Baysse, C., Anjaiah, V. L. & Cornelis, P. (2000). Quinolobactin, a new siderophore of *Pseudomonas fluorescens* ATCC 17400, the production of which is repressed by the cognate pyoverdine. *Appl Environ Microbiol* **66**, 487-492.

Ochsner, U. A. & Vasil, M. L. (1996). Gene repression by the ferric uptake regulator in *Pseudomonas aeruginosa*: cycle selection of iron-regulated genes. *Proc Natl Acad Sci U S A* **93**, 4409-4414.

Pessi, G., Williams, F., Hindle, Z., Heurlier, K., Holden, M. T. G., Camara, M., Haas, D. & Williams, P. (2001). The global posttranscriptional regulator RsmA modulates production of virulence determinants and *N*-acylhomoserine lactones in *Pseudomonas aeruginosa*. *J Bacteriol* **183**, 6676-6683.

Rahme, L. G., Stevens, E. J., Wolfort, S. F., Shao, J., Tompkins, R. G. & Ausubel, F. M. (1995). Common virulence factors for bacterial pathogenicity in plants and animals. *Science* **268**, 1899-1902.

Redly, G. A. & Poole, K. (2003). Pyoverdine-mediated regulation of FpvA synthesis in *Pseudomonas aeruginosa*: involvement of a probable extracytoplasmic-function sigma factor, FpvI. *J Bacteriol* **185**, 1261-1265.

Reimmann, C., Beyeler, M., Latifi, A., Winteler, H., Foglino, M., Lazdunski, A. & Haas, D. (1997). The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer N-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide, and lipase. *Mol Microbiol* **24**, 309-319.

Reimmann, C., Valverde, C., Kay, E. & Haas, D. (2005). Posttranscriptional repression of GacS/GacA-controlled genes by the RNA-binding protein RsmE acting together with RsmA in biocontrol strain *Pseudomonas fluorescens* CHA0. *J Bacteriol* **187**, 276-285.

Rich, J. J., Kinscherf, T. G., Kitten, T. & Willis, D. K. (1994). Genetic evidence that the *gacA* gene encodes the cognate response regulator for the *lemA* sensor in *Pseudomonas syringae*. *J Bacteriol* **176**, 7468-7475.

Sacherer, P., Defago, G. & Haas, D. (1994). Extracellular protease and phospholipase-C are controlled by the global regulatory gene *gacA* in the biocontrol strain *Pseudomonas fluorescens* CHA0. *FEMS Microbiol Lett* **116**, 155-160.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sánchez-Contreras, M., Martín, M., Villacieros, M., O'Gara, F., Bonilla, I. & Rivilla, R. (2002). Phenotypic selection and phase variation occur during alfalfa root colonization by *Pseudomonas fluorescens* F113. *J Bacteriol* **184**, 1587-1596.

Scher, F. M. & Baker, R. (1982). Effects of *Pseudomonas putida* and a synthetic iron chelator on induction of soil suppressiveness to *Fusarium wilt* pathogens. *Phytopathology* **72**, 1567-1573.

Shanahan, P., O´Sullivan, D. J., Simpson, P., Glennon, J. D. & O´Gara, F. (1992). Isolation of 2,4-diacetylphloroglucinol from a fluorescent pseudomonad and investigation of physiological parameters influencing its production. *Appl Environ Microbiol* **58**, 353-358.

Tart, A. H., Wolfgang, M. C. & Wozniak, D. J. (2005). The alternative sigma factor AlgT represses *Pseudomonas aeruginosa* flagellum biosynthesis by inhibiting expression of *fleQ*. *J Bacteriol* **187**, 7955-7962.

Valverde, C., Lindell, M., Wagner, E. G. H. & Haas, D. (2004). A repeated GGA motif is critical for the activity and stability of the riboregulator *rsmY* of *Pseudomonas fluorescens*. *J Biol Chem* **279**, 25066-25074.

Visca, P., Leoni, L., Wilson, M. J. & Lamont, I. L. (2002). Iron transport and regulation, cell signalling and genomics: lessons from *Escherichia coli* and *Pseudomonas*. *Mol Microbiol* **45**, 1177-1190.

Vodovar, N., Vallenet, D., Cruveiller, S. & other authors (2006). Complete genome sequence of the entomopathogenic and metabolically versatile soil bacterium *Pseudomonas entomophila*. *Nat Biotechnol* **24**, 673-679.

Wei, B. D. L., Brun-Zinkernagel, A. M., Simecka, J. W., Pruss, B. M., Babitzke, P. & Romeo, T. (2001). Positive regulation of motility and *flhDC* expression by the RNA-binding protein CsrA of *Escherichia coli*. *Mol Microbiol* **40**, 245-256.

Whistler, C. A., Corbell, N. A., Sarniguet, A., Ream, W. & Loper, J. E. (1998). The two-component regulators GacS and GacA influence accumulation of the stationary-phase sigma factor σ^s and the stress response in *Pseudomonas fluorescens* Pf-5. *J Bacteriol* **180**, 6635-6641.

Wilson, M. J., McMorran, B. J. & Lamont, I. L. (2001). Analysis of promoters recognized by PvdS, an extracytoplasmic-function sigma factor protein from *Pseudomonas aeruginosa*. *J Bacteriol* **183**, 2151-2155.

Willis, D. K., Holmstadt, J. J. & Kinscherf, T. G. (2001). Genetic evidence that loss of virulence associated with *gacS* or *gacA* mutations in *Pseudomonas syringae* B728a does not result from effects on alginate production. *Appl Environ Microbiol* **67**, 1400-1403.

Zhang, Z. G. & Pierson, L. S. (2001). A second quorum-sensing system regulates cell surface properties but not phenazine antibiotic production in *Pseudomonas aureofaciens*. *Appl Environ Microbiol* **67**, 4305-4315.

Zuber, S., Carruthers, F., Keel, C. & other authors (2003). GacS sensor domains pertinent to the regulation of exoproduct formation and to the biocontrol potential of *Pseudomonas fluorescens* CHA0. *Mol Plant-Microbe Interact* **16**, 634-644.

CONCLUSIONES

1. La organización genética de la región implicada en la síntesis del filamento flagelar de *P. fluorescens* F113 es similar a otras *Pseudomonas* que contienen flagelina tipo b pero difiere de la de *Pseudomonas* que contienen flagelina tipo a. El gen *fliC* codifica una flagelina tipo b. El gen *fleQ* es necesario para la producción y secreción de la flagelina FliC. La proteína FlaG regula la longitud del filamento flagelar. Además, afecta a la movilidad de tipo *swimming* de *P. fluorescens* F113 en medio rico o suplementado con hierro. La proteína FliS muestra características típicas de la mayoría de chaperonas citoplasmáticas y esta implicada en la estabilización y secreción de la flagelina FliC. El gen *fliT* afecta a la movilidad de tipo *swimming* de *P. fluorescens* F113.
2. Los mutantes inmóviles o con una movilidad reducida no son buenos colonizadores competitivos y son desplazados de la rizosfera por la cepa silvestre.
3. Las recombinasas específicas de sitio Sss y XerD son las principales responsables de la variación fenotípica de *P. fluorescens* F113 tanto en fase estacionaria como durante la colonización de la rizosfera de alfalfa, siendo necesarias para la colonización competitiva de la rizosfera de alfalfa por *P. fluorescens* F113.
4. Los genes *sss* y *xerD* se inducen en fase estacionaria y en la rizosfera de alfalfa. El inductor en la rizosfera de alfalfa es un compuesto no difusible presente en la raíz.
5. Los variantes de fase (fenotípicos) procedentes de la sobreexpresión de las recombinasas Sss y XerD están afectados en el sistema de dos componentes GacA/GacS.
6. Durante la colonización de la rizosfera de alfalfa aparecen variantes que presentan mayor movilidad que los variantes procedentes de experimentos realizados en cultivo líquido de larga duración. Esta característica es una consecuencia de mutaciones adicionales independientes del sistema

GacA/GacS. Algunos de estos variantes son más competitivos que la cepa silvestre en ensayos de colonización de la raíz de alfalfa.

7. El sistema GacA/GacS regula negativamente la movilidad de tipo *swimming* y la expresión del gen *pvdA*, que está implicado en la producción de pioverdina, a través de las proteínas RsmA y RsmE en *P. fluorescens* F113. Este sistema de dos componentes también regula negativamente la movilidad de tipo *swimming* y la producción de pioverdina en *P. fluorescens* Pf0-1.
8. La regulación de la movilidad a través del sistema GacA/GacS afecta a la expresión del gen que codifica la proteína reguladora FleQ. A su vez la expresión de *fleQ* afecta a la expresión del gen *fliC* que conlleva un cambio en la producción de flagelina. La represión de *fleQ* y de la producción de pioverdina a través del sistema GacA/GacS es independiente de Vfr y Fur, respectivamente.

ANEXO I

Secuencias

Secuencia gen *fliC*

```
    ---------|---------|---------|---------|---------|---------|
  1 atggctttaacagtaaacactaacgtcacatcgttgaacgttcagaagaacctgaacaag 60
  1 M  A  L  T  V  N  T  N  V  T  S  L  N  V  Q  K  N  L  N  K   20

    ---------|---------|---------|---------|---------|---------|
 61 gcttccgatgctctgtccacttcgatgacccgtctgtcttccggcctgaaaatcaacagc 120
 21 A  S  D  A  L  S  T  S  M  T  R  L  S  S  G  L  K  I  N  S   40

    ---------|---------|---------|---------|---------|---------|
121 gccaaagacgacgccgccggcttacagatcgctacccgtatgacttcgcaaatccgcggt 180
 41 A  K  D  D  A  A  G  L  Q  I  A  T  R  M  T  S  Q  I  R  G   60

    ---------|---------|---------|---------|---------|---------|
181 cagactgttgcgatcaagaacgccaacgacggtatctctatcgctcagaccgctgaaggc 240
 61 Q  T  V  A  I  K  N  A  N  D  G  I  S  I  A  Q  T  A  E  G   80

    ---------|---------|---------|---------|---------|---------|
241 gctctgcaggaatccaccaacatcctgcagcgtatgcgtgaactggctgtccaggctcga 300
 81 A  L  Q  E  S  T  N  I  L  Q  R  M  R  E  L  A  V  Q  A  R   100

    ---------|---------|---------|---------|---------|---------|
301 aacgactccaacggtactgctgaccgtgacgctctgaacaaagaatttgctcagatgtcg 360
101 N  D  S  N  G  T  A  D  R  D  A  L  N  K  E  F  A  Q  M  S   120

    ---------|---------|---------|---------|---------|---------|
361 gacgagctgacccgtatcgccgagtcgaccaacctgaacggcaagaacctgatcgacggt 420
121 D  E  L  T  R  I  A  E  S  T  N  L  N  G  K  N  L  I  D  G   140

    ---------|---------|---------|---------|---------|---------|
421 tccgctggcaccatgaccttccaggtcggttccaacaccggtgctaccaaccagatcact 480
141 S  A  G  T  M  T  F  Q  V  G  S  N  T  G  A  T  N  Q  I  T   160

    ---------|---------|---------|---------|---------|---------|
481 ctgaccctggatagcggcttcgacgctgcaaccttgagtgttgactctgccgccatcgcc 540
161 L  T  L  D  S  G  F  D  A  A  T  L  S  V  D  S  A  A  I  A   180

    ---------|---------|---------|---------|---------|---------|
541 atcaccggtaacagcagcgccactgccgaagctagcactgctgctgcaatcgacgcaatc 600
181 I  T  G  N  S  S  A  T  A  E  A  S  T  A  A  A  I  D  A  I   200

    ---------|---------|---------|---------|---------|---------|
601 gacgcagctctggcaaccatcaactccagccgtgctgacctcggtgctgcacaaaaccgt 660
201 D  A  A  L  A  T  I  N  S  S  R  A  D  L  G  A  A  Q  N  R   220

    ---------|---------|---------|---------|---------|---------|
661 ctgaccagcaccatctccaacctgcagaacgtcaacgaaaacgccgccgctgcactgggt 720
221 L  T  S  T  I  S  N  L  Q  N  V  N  E  N  A  A  A  A  L  G   240

    ---------|---------|---------|---------|---------|---------|
721 cgcgtacaagacaccgacttcgctgctgaaactgcccagctgaccaagcagcagactctg 780
241 R  V  Q  D  T  D  F  A  A  E  T  A  Q  L  T  K  Q  Q  T  L   260

    ---------|---------|---------|---------|---------|---------|
781 cagcaggcttccacttcggttctggcccaggccaaccaactgccatccgctgtactgaag 840
261 Q  Q  A  S  T  S  V  L  A  Q  A  N  Q  L  P  S  A  V  L  K   280

    ---------|--
841 ctgcttcagtaa 852
281 L  L  Q  *   283
```

Secuencia gen *flaG*

```
    ---------|---------|---------|---------|---------|---------|
  1 atgagcgtgaagctgaacttgtcttatccagctgcgaagccggcgacgacatttgccgac 60
  1 M  S  V  K  L  N  L  S  Y  P  A  A  K  P  A  T  T  F  A  D   20

    ---------|---------|---------|---------|---------|---------|
 61 aaaccagtggagaagcctcgagccgatgctgccaccgtggtaccggtcaaggatgagccg 120
 21 K  P  V  E  K  P  R  A  D  A  A  T  V  V  P  V  K  D  E  P   40

    ---------|---------|---------|---------|---------|---------|
121 aaagacgctgtggccgagcaagagaaactgaagagggcggttcaggaaattgaaaccttc 180
 41 K  D  A  V  A  E  Q  E  K  L  K  R  A  V  Q  E  I  E  T  F   60

    ---------|---------|---------|---------|---------|---------|
181 gttcagtcggtcaagcgcaacctggagttctcaatcgatgagccttcaggcaaagtagtt 240
 61 V  Q  S  V  K  R  N  L  E  F  S  I  D  E  P  S  G  K  V  V   80

    ---------|---------|---------|---------|---------|---------|
241 gtcaaagtgattgccagtggttccggtgaagtgattcgccagatccctaatgaagaagtg 300
 81 V  K  V  I  A  S  G  S  G  E  V  I  R  Q  I  P  N  E  E  V  100

    ---------|---------|---------|---------|---------|---------|
301 cttaaactggcgaacagtttgaatgatgcaagcagcctgttgttcagcgcgcaagcctga 360
101 L  K  L  A  N  S  L  N  D  A  S  S  L  L  F  S  A  Q  A  *  119
```

Secuencia gen *fliD*

```
    ---------|---------|---------|---------|---------|---------|
  1 atggtcaaaagggccggcgacacacttgaagggagttccacatggcaaggtccaattcta 60
  1 M  V  K  R  A  G  D  T  L  E  G  S  S  T  W  Q  G  P  I  L   20

    ---------|---------|---------|---------|---------|---------|
 61 cctggcttgggtctaggctctggccttgataccggtgctatcgtcaaggcattggtggat 120
 21 P  G  L  G  L  G  S  G  L  D  T  G  A  I  V  K  A  L  V  D   40

    ---------|---------|---------|---------|---------|---------|
121 tctgacaaagcagccaagcaaggtcagattgaccgtgccaccaagaccaatacgggcaat 180
 41 S  D  K  A  A  K  Q  G  Q  I  D  R  A  T  K  T  N  T  G  N   60

    ---------|---------|---------|---------|---------|---------|
181 atttcggggatcggcacccttaaatcgctgctggctacctttcagtctgcgcttgaaagc 240
 61 I  S  G  I  G  T  L  K  S  L  L  A  T  F  Q  S  A  L  E  S   80

    ---------|---------|---------|---------|---------|---------|
241 ctgggcagcacgaccacccctcaatttaccggcgttgcagccacttcggccaataccagt 300
 81 L  G  S  T  T  T  P  Q  F  T  G  V  A  A  T  S  A  N  T  S  100

    ---------|---------|---------|---------|---------|---------|
301 gcgttgacggtgacggccggaaattcggcagttggtggtatttactcagtagacgttact 360
101 A  L  T  V  T  A  G  N  S  A  V  G  G  I  Y  S  V  D  V  T  120

    ---------|---------|---------|---------|---------|---------|
361 cagctggcgacctcctcgaaagttgcgacggctgctttcgcaggtggcgcttccagtgcg 420
121 Q  L  A  T  S  S  K  V  A  T  A  A  F  A  G  G  A  S  S  A  140
```

```
    ---------|---------|---------|---------|---------|---------|
 421 gtggcgaccggtacgctgaccatcagtcagggaggcacggattacacccttgatatccct 480
 141 V  A  T  G  T  L  T  I  S  Q  G  G  T  D  Y  T  L  D  I  P   160

    ---------|---------|---------|---------|---------|---------|
 481 gctgattcgacgctccaatcggttcgggatgctatcaatagtaaatactcctccagtggt 540
 161 A  D  S  T  L  Q  S  V  R  D  A  I  N  S  K  Y  S  S  S  G   180

    ---------|---------|---------|---------|---------|---------|
 541 ctgaccgccaacatcgtgaccgacagtttcggttcgcgtcttgtggtggggtcgactaag 600
 181 L  T  A  N  I  V  T  D  S  F  G  S  R  L  V  V  G  S  T  K   200

    ---------|---------|---------|---------|---------|---------|
 601 accggggcgggtaacgatatttctcttagtggcattgccagccttgcagctgacggctcc 660
 201 T  G  A  G  N  D  I  S  L  S  G  I  A  S  L  A  A  D  G  S   220

    ---------|---------|---------|---------|---------|---------|
 661 gtagcgatggcttcaccacccactgcgaattcttcaggttcgctggggtttgccaaggac 720
 221 V  A  M  A  S  P  P  T  A  N  S  S  G  S  L  G  F  A  K  D   240

    ---------|---------|---------|---------|---------|---------|
 721 gccattttcagtgttgacggcctggaaatgaccagtccgaccaacaagctggataatgtg 780
 241 A  I  F  S  V  D  G  L  E  M  T  S  P  T  N  K  L  D  N  V   260

    ---------|---------|---------|---------|---------|---------|
 781 gtctccggcttgagtatgacgttgcttgttgccgataccggcccaacgaccgtcaccgta 840
 261 V  S  G  L  S  M  T  L  L  V  A  D  T  G  P  T  T  V  T  V   280

    ---------|---------|---------|---------|---------|---------|
 841 gccgctaacaccgatggcctgaaagcttcgattcagaaatttgtcgatgcttataacgcc 900
 281 A  A  N  T  D  G  L  K  A  S  I  Q  K  F  V  D  A  Y  N  A   300

    ---------|---------|---------|---------|---------|---------|
 901 atcgcgaaggccgtgacttccctgaccaagccatcgacagatgccgaaggtaactcggtt 960
 301 I  A  K  A  V  T  S  L  T  K  P  S  T  D  A  E  G  N  S  V   320

    ---------|---------|---------|---------|---------|---------|
 961 ccggcagcattgacgggcgactcattgcctcgctcgcttttggcggccattcgcgcgcct 1020
 321 P  A  A  L  T  G  D  S  L  P  R  S  L  L  A  A  I  R  A  P   340

    ---------|---------|---------|---------|---------|---------|
1021 ttgtccgaaaccggtgctggcgataagctgaccgtgttgtctcagttggggatcacgacc 1080
 341 L  S  E  T  G  A  G  D  K  L  T  V  L  S  Q  L  G  I  T  T   360

    ---------|---------|---------|---------|---------|---------|
1081 aaccagacgacgggcgccctggattttgacagtacaaagttcgcaactgccgtgaacgaa 1140
 361 N  Q  T  T  G  A  L  D  F  D  S  T  K  F  A  T  A  V  N  E   380

    ---------|---------|---------|---------|---------|---------|
1141 aaacagttgggtggagaaatccagaccctgttcacaggcgagaatggcctgatcgagcgc 1200
 381 K  Q  L  G  G  E  I  Q  T  L  F  T  G  E  N  G  L  I  E  R   400

    ---------|---------|---------|---------|---------|---------|
1201 atgcagaatgcactcaatccccacacggagaccagcaagaaggactccaatgggaagacc 1260
 401 M  Q  N  A  L  N  P  H  T  E  T  S  K  K  D  S  N  G  K  T   420

    ---------|---------|---------|---------|---------|---------|
1261 gtcgataacatcctgacggcgcgctccaagaatcttgaaattctcaaggcgaaactgagc 1320
 421 V  D  N  I  L  T  A  R  S  K  N  L  E  I  L  K  A  K  L  S   440
```

```
    ---------|---------|---------|---------|---------|---------|
1321 gaggaccagttggccctggaccgccgcatcgaaaccctcaccgccgtactgaccaaaaag 1380
 441 E  D  Q  L  A  L  D  R  R  I  E  T  L  T  A  V  L  T  K  K   460

    ---------|---------|---------|---------|---------|---------|
1381 tacaacgacatggacaccctggtaggcaggctgaaagccaccgccagtaacatcacctcc 1440
 461 Y  N  D  M  D  T  L  V  G  R  L  K  A  T  A  S  N  I  T  S   480

    ---------|---------|---------|---------
1441 atgttcgaagcgatgacggcacagcagaaaaacagctga 1479
 481 M  F  E  A  M  T  A  Q  Q  K  N  S  *   492
```

Secuencia gen *fliS*

```
    ---------|---------|---------|---------|---------|---------|
   1 atgaatccgatgttagcccctcgccaataccagaagattggcgcccaggcgcaaacctcc 60
   1 M  N  P  M  L  A  P  R  Q  Y  Q  K  I  G  A  Q  A  Q  T  S   20

    ---------|---------|---------|---------|---------|---------|
  61 gaagcaagtccccatcgtctggtgcagatgctcatggaaggcgggctggatcgcattgcc 120
  21 E  A  S  P  H  R  L  V  Q  M  L  M  E  G  G  L  D  R  I  A   40

    ---------|---------|---------|---------|---------|---------|
 121 caggccaagggtgcgatggagcgcaaggatatcgccaacaaaggcgtactgatcagcaag 180
  41 Q  A  K  G  A  M  E  R  K  D  I  A  N  K  G  V  L  I  S  K   60

    ---------|---------|---------|---------|---------|---------|
 181 gccatcggcattatcggcggtttgcgtgaaggcctggacctggaaaaccaggccgagtcg 240
  61 A  I  G  I  I  G  G  L  R  E  G  L  D  L  E  N  Q  A  E  S   80

    ---------|---------|---------|---------|---------|---------|
 241 gtaaccgagctggataacctctacacctacatgatgaaacgcctggccgaggccaacgcc 300
  81 V  T  E  L  D  N  L  Y  T  Y  M  M  K  R  L  A  E  A  N  A   100

    ---------|---------|---------|---------|---------|---------|
 301 aagaccgatccgaagatcctcgacgaagtcgccgatctgcttggcacggtcaaggaaggt 360
 101 K  T  D  P  K  I  L  D  E  V  A  D  L  L  G  T  V  K  E  G   120

    ---------|---------|---------|------
 361 tgggatgccatcgctgtaccgggtccgcaattctaa 396
 121 W  D  A  I  A  V  P  G  P  Q  F  *   131
```

Secuencia gen *fliT*

```
    ---------|---------|---------|---------|---------|---------|
   1 atgagtcttgtcttgcagcgaatcgaacaaacccgtgatgccttggtcgatgccctggct 60
   1 M  S  L  V  L  Q  R  I  E  Q  T  R  D  A  L  V  D  A  L  A   20

    ---------|---------|---------|---------|---------|---------|
  61 gagcgtaactgggaggccatcggtcagttggacctggcctgccgttcctgcatggaagac 120
  21 E  R  N  W  E  A  I  G  Q  L  D  L  A  C  R  S  C  M  E  D   40

    ---------|---------|---------|---------|---------|---------|
 121 gtcctgagcgagtctcaggtggatgaggcggcgttgcggatcaatcttgaggaattgctg 180
  41 V  L  S  E  S  Q  V  D  E  A  A  L  R  I  N  L  E  E  L  L   60
```

```
    ---------|---------|---------|---------|---------|---------|
181 ggggtgtatcggcaattgctggaggcagcgactggagagcgtcaggcgatcgtcgacgag 240
 61 G  V  Y  R  Q  L  L  E  A  A  T  G  E  R  Q  A  I  V  D  E   80

    ---------|---------|---------|---------|---------|-------
241 atgcagcagatccatcaagcacagaacgctgcaaaggtttaccatctgttcggttaa 297
 81 M  Q  Q  I  H  Q  A  Q  N  A  A  K  V  Y  H  L  F  G  *   98
```

Secuencia gen *fleQ*

```
    ---------|---------|---------|---------|---------|---------|
  1 atgtggcgtgaaaccaaaattctcctgatcgatgacgatagcgtccgccgccgcgacttg 60
  1 M  W  R  E  T  K  I  L  L  I  D  D  D  S  V  R  R  R  D  L   20

    ---------|---------|---------|---------|---------|---------|
 61 gcggtgatcttaaattttcttggcgaagaaaatttaccctgcggaagccatgactggcag 120
 21 A  V  I  L  N  F  L  G  E  E  N  L  P  C  G  S  H  D  W  Q   40

    ---------|---------|---------|---------|---------|---------|
121 caggccgtcggctctttgtcgtccagtcgcgaggtcatttgtgtccttatcgggaccgtg 180
 41 Q  A  V  G  S  L  S  S  S  R  E  V  I  C  V  L  I  G  T  V   60

    ---------|---------|---------|---------|---------|---------|
181 aatgcccccgcaacgcttttgggcttgctaaagacactctcaacctgggatgagttcctt 240
 61 N  A  P  A  T  L  L  G  L  L  K  T  L  S  T  W  D  E  F  L   80

    ---------|---------|---------|---------|---------|---------|
241 ccggttttgttaatgggcgaaaattcttcccttgacttgcctgaggaccagcgccgccgg 300
 81 P  V  L  L  M  G  E  N  S  S  L  D  L  P  E  D  Q  R  R  R  100

    ---------|---------|---------|---------|---------|---------|
301 gtactttccacgctcgaaatgccgcccagctacagcaagctgcttgattcgctgcaccgc 360
101 V  L  S  T  L  E  M  P  P  S  Y  S  K  L  L  D  S  L  H  R  120

    ---------|---------|---------|---------|---------|---------|
361 gcgcaggtttatcgcgagatgtacgatcaggctcgcgagcgcggccggcatcgcgaaccc 420
121 A  Q  V  Y  R  E  M  Y  D  Q  A  R  E  R  G  R  H  R  E  P  140

    ---------|---------|---------|---------|---------|---------|
421 aacctgttccgcagcctcgtcggcaccagccgggcgatccagcacgtgcggcagatgatg 480
141 N  L  F  R  S  L  V  G  T  S  R  A  I  Q  H  V  R  Q  M  M  160

    ---------|---------|---------|---------|---------|---------|
481 cagcaggtcgccgataccgacgccagcgtgctgatcctgggtgagtcgggcaccggcaag 540
161 Q  Q  V  A  D  T  D  A  S  V  L  I  L  G  E  S  G  T  G  K  180

    ---------|---------|---------|---------|---------|---------|
541 gaagtggttgcgcgcaacctgcattaccactccaagcgccgcgaagcgccgttcgtgcca 600
181 E  V  V  A  R  N  L  H  Y  H  S  K  R  R  E  A  P  F  V  P  200

    ---------|---------|---------|---------|---------|---------|
601 gtcaactgtggcgcgatcccggccgagttgctggagagcgagttgtttggccatgagaag 660
201 V  N  C  G  A  I  P  A  E  L  L  E  S  E  L  F  G  H  E  K  220

    ---------|---------|---------|---------|---------|---------|
661 ggcgcctttaccggcgcgatcaccagtcgcgccgggcgtttcgaactggccaatggcggt 720
221 G  A  F  T  G  A  I  T  S  R  A  G  R  F  E  L  A  N  G  G  240
```

```
    ---------|---------|---------|---------|---------|---------|
 721 acgctgttcctcgacgaaatcggtgacatgccgctgccgatgcaggtcaagctgttgcgg 780
 241 T  L  F  L  D  E  I  G  D  M  P  L  P  M  Q  V  K  L  L  R   260

    ---------|---------|---------|---------|---------|---------|
 781 gtcctgcaggagcgcaccttcgagcgcgtgggcagcaacaagacccagagcgtcgatgtg 840
 261 V  L  Q  E  R  T  F  E  R  V  G  S  N  K  T  Q  S  V  D  V   280

    ---------|---------|---------|---------|---------|---------|
 841 cgcatcatcgccgccacgcacaagaacctcgagagcatgatcgaggttggcagcttccgc 900
 281 R  I  I  A  A  T  H  K  N  L  E  S  M  I  E  V  G  S  F  R   300

    ---------|---------|---------|---------|---------|---------|
 901 gaagacctctattaccgcctcaacgttttcccgatcgaaatggcgccactgcgtgagcgc 960
 301 E  D  L  Y  Y  R  L  N  V  F  P  I  E  M  A  P  L  R  E  R   320

    ---------|---------|---------|---------|---------|---------|
 961 gtcgaagacatcccgttgctgatgaacgagctgatctcgcgcatggagcacgaaaagcgc 1020
 321 V  E  D  I  P  L  L  M  N  E  L  I  S  R  M  E  H  E  K  R   340

    ---------|---------|---------|---------|---------|---------|
1021 ggttcgatccgcttcaattcggcggcgatcatgtccctgtgccgtcatggctggccgggc 1080
 341 G  S  I  R  F  N  S  A  A  I  M  S  L  C  R  H  G  W  P  G   360

    ---------|---------|---------|---------|---------|---------|
1081 aacgtccgggaactggccaacctggtggagcgcatggcgatcatgcacccgtacggggtg 1140
 361 N  V  R  E  L  A  N  L  V  E  R  M  A  I  M  H  P  Y  G  V   380

    ---------|---------|---------|---------|---------|---------|
1141 atcggcgtgaacgaactgccgaagaaattccgctacgtcgacgacgaagacgagcaaatg 1200
 381 I  G  V  N  E  L  P  K  K  F  R  Y  V  D  D  E  D  E  Q  M   400

    ---------|---------|---------|---------|---------|---------|
1201 gtcgacagcctgcgcagcgatctggaagagcgggtggcaatcaacggccacacaccggat 1260
 401 V  D  S  L  R  S  D  L  E  E  R  V  A  I  N  G  H  T  P  D   420

    ---------|---------|---------|---------|---------|---------|
1261 ttcaccgccaatgcgttgttgccgcccgaaggcctggacctcaaggattacctgggaggc 1320
 421 F  T  A  N  A  L  L  P  P  E  G  L  D  L  K  D  Y  L  G  G   440

    ---------|---------|---------|---------|---------|---------|
1321 ctggagcaagggctgatccagcaggcgctggacgatgccaacggtatcgttgcccgcgcg 1380
 441 L  E  Q  G  L  I  Q  Q  A  L  D  D  A  N  G  I  V  A  R  A   460

    ---------|---------|---------|---------|---------|---------|
1381 gcagaacgcctgcgcatccgccgtacctccctggtggaggagatgcgcaagtacggcatg 1440
 461 A  E  R  L  R  I  R  R  T  S  L  V  E  E  M  R  K  Y  G  M   480

    ---------|---------|---------|------
1441 agccgccgtgaaggagatgaacaggcggatgattga 1476
 481 S  R  R  E  G  D  E  Q  A  D  D  *   491
```

Secuencia parcial gen *fleS*

```
    ---------|---------|---------|---------|---------|---------|
   1 atgaccctagccgcccagatgtctcctgtccccgagccggaacacatgccgtccgccgag 60
   1 M  T  L  A  A  Q  M  S  P  V  P  E  P  E  H  M  P  S  A  E   20
```

```
    ---------|---------|---------|---------|---------|---------|
 61 caggcaagccggcttggacttgagcaagcattttcgctgttcaaccagatgtccagtcaa 120
 21 Q  A  S  R  L  G  L  E  Q  A  F  S  L  F  N  Q  M  S  S  Q   40

    ---------|---------|---------|---------|---------|---------|
121 ctgacagactcctacagcctgcttgaagcccgggttaccgagctcaagggtgagctggcc 180
 41 L  T  D  S  Y  S  L  L  E  A  R  V  T  E  L  K  G  E  L  A   60

    ---------|---------|---------|---------|---------|---------|
181 gtggtcagcgcccagcgcatgcaggagcttgcggaaaaggaacgcctggctaatcgtctg 240
 61 V  V  S  A  Q  R  M  Q  E  L  A  E  K  E  R  L  A  N  R  L   80

    ---------|---------|---------|---------|---------|---------|
241 caaaatctcctcgacctgttgcctggtggcgttatcgtcatcgacgcccacggccgtgtg 300
 81 Q  N  L  L  D  L  L  P  G  G  V  I  V  I  D  A  H  G  R  V   100

    ---------|---------|---------|---------|---------|---------|
301 cgcgaagccaacccggcggcttgcgagttgctcggcctgccgttggaaggtgagctgtgg 360
101 R  E  A  N  P  A  A  C  E  L  L  G  L  P  L  E  G  E  L  W   120

    ---------|---------|---------|---------|---------|---------|
361 cggcatgtcatcgcccgctgctttgcgccccgtgaagacgacggccatgaagtgtccctc 420
121 R  H  V  I  A  R  C  F  A  P  R  E  D  D  G  H  E  V  S  L   140

    ---------|---------|---------|---------|---------|---------|
421 aaggacggtcggcgcctgtccatctcgacacgttcgctggatgccgagccggggcagttg 480
141 K  D  G  R  R  L  S  I  S  T  R  S  L  D  A  E  P  G  Q  L   160

    ---------|---------|---------|---------|---------|---------|
481 gtgctgctcaatgacttgactgaaacccgtcacctgcaagaccagttggctcgccatgag 540
161 V  L  L  N  D  L  T  E  T  R  H  L  Q  D  Q  L  A  R  H  E   180

    ---------|---------|---------|---------|---------|---------|
541 cgcctgtcttccctggggcggatggtggcgtcgctggcccatcagattcgtacgccgctt 600
181 R  L  S  S  L  G  R  M  V  A  S  L  A  H  Q  I  R  T  P  L   200

    ---------|---------|---------|---------|---------|---------|
601 tctgccgcgctgctctatgccagtcatctggctgaacagcaattgccgatggacacccag 660
201 S  A  A  L  L  Y  A  S  H  L  A  E  Q  Q  L  P  M  D  T  Q   220

    ---------|---------|---------|---------|---------|---------|
661 caacggttcgccggacgcttgaaagagcgcctgcatgagctggagcaccaggtgcgcgac 720
221 Q  R  F  A  G  R  L  K  E  R  L  H  E  L  E  H  Q  V  R  D   240

    ---------|------
721 atgctggtattcaatc 736
241 M  L  V  F  N    245
```

Región síntesis del filamento flagelar

ACCESSION AF399739

gene="fliC"
CDS 353..1204
product="flagellin"
protein_id="AAL57341.1"
db_xref="GI:18034057"
translation="MALTVNTNVTSLNVQKNLNKASDALSTSMTRLSSGLKINSAKD
DAAGLQIATRMTSQIRGQTVAIKNANDGISIAQTAEGALQESTNILQRMREL
AVQARNDSNGTADRDALNKEFAQMSDELTRIAESTNLNGKNLIDGSAGTM
TFQVGSNTGATNQITLTLDSGFDAATLSVDSAAIAITGNSSATAEASTAAAI
DAIDAALATINSSRADLGAAQNRLTSTISNLQNVNENAAAALGRVQDTDFA
AETAQLTKQQTLQQASTSVLAQANQLPSAVLKLLQ"

gene="flaG"
CDS 1283..1642
product="FlaG"
translation="MSVKLNLSYPAAKPATTFADKPVEKPRADAATVVPVKDEPKD
AVAEQEKLKRAVQEIETFVQSVKRNLEFSIDEPSGKVVVKVIASGSGEVIRQI
PNEEVLKLANSLNDASSLLFSAQA"

gene="fliD"
CDS 1688..3166
note="flagellar cap protein"
product="FliD"
translation="MVKRAGDTLEGSSTWQGPILPGLGLGSGLDTGAIVKALVDSDK
AAKQGQIDRATKTNTGNISGIGTLKSLLATFQSALESLGSTTTPQFTGVAAT
SANTSALTVTAGNSAVGGIYSVDVTQLATSSKVATAAFAGGASSAVATGTL
TISQGGTDYTLDIPADSTLQSVRDAINSKYSSSGLTANIVTDSFGSRLVVGST
KTGAGNDISLSGIASLAADGSVAMASPPTANSSGSLGFAKDAIFSVDGLEMT
SPTNKLDNVVSGLSMTLLVADTGPTTVTVAANTDGLKASIQKFVDAYNAIA
KAVTSLTKPSTDAEGNSVPAALTGDSLPRSLLAAIRAPLSETGAGDKLTVLS
QLGITTNQTTGALDFDSTKFATAVNEKQLGGEIQTLFTGENGLIERMQNAL
NPHTETSKKDSNGKTVDNILTARSKNLEILKAKLSEDQLALDRRIETLTAVL
TKKYNDMDTLVGRLKATASNITSMFEAMTAQQKNS"

gene="fliS"
CDS 3298..3693
note="putative cytosolic export chaperone"
product="FliS"

translation="MNPMLAPRQYQKIGAQAQTSEASPHRLVQMLMEGGLDRIAQA
KGAMERKDIANKGVLISKAIGIIGGLREGLDLENQAESVTELDNLYTYMMK
RLAEANAKTDPKILDEVADLLGTVKEGWDAIAVPGPQF"

gene="fliT"
CDS 3705..4001
product="FliT"
translation="MSLVLQRIEQTRDALVDALAERNWEAIGQLDLACRSCMEDVL
SESQVDEAALRINLEELLGVYRQLLEAATGERQAIVDEMQQIHQAQNAAK
VYHLFG"

gene="fleQ"
CDS 4171..5646
note="flagellar regulatory protein"
product="FleQ"
translation="MWRETKILLIDDDSVRRRDLAVILNFLGEENLPCGSHDWQQAV
GSLSSSREVICVLIGTVNAPATLLGLLKTLSTWDEFLPVLLMGENSSLDLPED
QRRRVLSTLEMPPSYSKLLDSLHRAQVYREMYDQARERGRHREPNLFRSL
VGTSRAIQHVRQMMQQVADTDASVLILGESGTGKEVVARNLHYHSKRREA
PFVPVNCGAIPAELLESELFGHEKGAFTGAITSRAGRFELANGGTLFLDEIGD
MPLPMQVKLLRVLQERTFERVGSNKTQSVDVRIIAATHKNLESMIEVGSFR
EDLYYRLNVFPIEMAPLRERVEDIPLLMNELISRMEHEKRGSIRFNSAAIMSL
CRHGWPGNVRELANLVERMAIMHPYGVIGVNELPKKFRYVDDEDEQMVD
SLRSDLEERVAINGHTPDFTANALLPPEGLDLKDYLGGLEQGLIQQALDDA
NGIVARAAERLRIRRTSLVEEMRKYGMSRREGDEQADD"

gene="fleS"
CDS 5757..>6492
note="two-component system sensor protein"
product="FleS"
translation="MTLAAQMSPVPEPEHMPSAEQASRLGLEQAFSLFNQMSSQLTD
SYSLLEARVTELKGELAVVSAQRMQELAEKERLANRLQNLLDLLPGGVIVI
DAHGRVREANPAACELLGLPLEGELWRHVIARCFAPREDDGHEVSLKDGR
RLSISTRSLDAEPGQLVLLNDLTETRHLQDQLARHERLSSLGRMVASLAHQI
RTPLSAALLYASHLAEQQLPMDTQQRFAGRLKERLHELEHQVRDMLVFN"

```
  1 tgatgttcaa cgatcagcag gctcagaact ggctctcgct ggcggactat cttgagtacg
 61 aatgggttga gctgttgacc gctggtcagg cggtgtaatt ccttttcttg atgagctggc
121 gccgctgccc aagcgaatcg cgcccccgac atttccctgg cgtcattttt ttacctgtcg
181 agcgctgcca agtccttgat ttacgagggg tggcaccctg atggcaaaat ttttgaaaaa
241 actgctaaag caagttccga ttacgacgat aactattacg aaggttctct aggccacacc
301 cggcggttgc cagggccgga agccgcagta cccaaccaac gaggaattcg tcatggcttt
361 aacagtaaac actaacgtca catcgttgaa cgttcagaag aacctgaaca aggcttccga
```

421 tgctctgtcc acttcgatga cccgtctgtc ttccggcctg aaaatcaaca gcgccaaaga
481 cgacgccgcc ggcttacaga tcgctacccg tatgacttcg caaatccgcg gtcagactgt
541 tgcgatcaag aacgccaacg acggtatctc tatcgctcag accgctgaag gcgctctgca
601 ggaatccacc aacatcctgc agcgtatgcg tgaactggct gtccaggctc gaaacgactc
661 caacggtact gctgaccgtg acgctctgaa caaagaattt gctcagatgt cggacgagct
721 gacccgtatc gccgagtcga ccaacctgaa cggcaagaac ctgatcgacg gttccgctgg
781 caccatgacc ttccaggtcg gttccaacac cggtgctacc aaccagatca ctctgaccct
841 ggatagcggc ttcgacgctg caaccttgag tgttgactct gccgccatcg ccatcaccgg
901 taacagcagc gccactgccg aagctagcac tgctgctgca atcgacgcaa tcgacgcagc
961 tctggcaacc atcaactcca gccgtgctga cctcggtgct gcacaaaacc gtctgaccag
1021 caccatctcc aacctgcaga acgtcaacga aaacgccgcc gctgcactgg gtcgcgtaca
1081 agacaccgac ttcgctgctg aaactgccca gctgaccaag cagcagactc tgcagcaggc
1141 ttccacttcg gttctggccc aggccaacca actgccatcc gctgtactga agctgcttca
1201 gtaatagccg gatgagtttt agcgggggag tgcgcttgcg tactctctcg ctttttccgt
1261 tcaagaggtg atggacatgg atatgagcgt gaagctgaac ttgtcttatc cagctgcgaa
1321 gccggcgacg acatttgccg acaaaccagt ggagaagcct cgagccgatg ctgccaccgt
1381 ggtaccggtc aaggatgagc cgaaagacgc tgtggccgag caagagaaac tgaagagggc
1441 ggttcaggaa attgaaacct tcgttcagtc ggtcaagcgc aacctggagt tctcaatcga
1501 tgagccttca ggcaaagtag ttgtcaaagt gattgccagt ggttccggtg aagtgattcg
1561 ccagatccct aatgaagaag tgcttaaact ggcgaacagt ttgaatgatg caagcagcct
1621 gttgttcagc gcgcaagcct gactgctggc accgaatttt gttgttaagt tcttttgggc
1681 gttggtaatg gtcaaaaggg ccggcgacac acttgaaggg agttccacat ggcaaggtcc
1741 aattctacct ggcttgggtc taggctctgg ccttgatacc ggtgctatcg tcaaggcatt
1801 ggtggattct gacaaagcag ccaagcaagg tcagattgac cgtgccacca agaccaatac
1861 gggcaatatt tcggggatcg gcacccttaa atcgctgctg gctacctttc agtctgcgct
1921 tgaaagcctg ggcagcacga ccacccctca atttaccggc gttgcagcca cttcggccaa
1981 taccagtgcg ttgacggtga cggccggaaa ttcggcagtt ggtggtattt actcagtaga
2041 cgttactcag ctggcgacct cctcgaaagt tgcgacggct gctttcgcag gtggcgcttc
2101 cagtgcggtg gcgaccggta cgctgaccat cagtcaggga ggcacggatt acacccttga
2161 tatccctgct gattcgacgc tccaatcggt tcgggatgct atcaatagta aatactcctc
2221 cagtggtctg accgccaaca tcgtgaccga cagtttcggt tcgcgtcttg tggtggggtc
2281 gactaagacc ggggcgggta acgatatttc tcttagtggc attgccagcc ttgcagctga
2341 cggctccgta gcgatggctt caccacccac tgcgaattct tcaggttcgc tggggtttgc
2401 caaggacgcc attttcagtg ttgacggcct ggaaatgacc agtccgacca acaagctgga
2461 taatgtggtc tccggcttga gtatgacgtt gcttgttgcc gataccggcc caacgaccgt
2521 caccgtagcc gctaacaccg atggcctgaa agcttcgatt cagaaatttg tcgatgctta
2581 taacgccatc gcgaaggccg tgacttccct gaccaagcca tcgacagatg ccgaaggtaa
2641 ctcggttccg gcagcattga cgggcgactc attgcctcgc tcgcttttgg cggccattcg
2701 cgcgcctttg tccgaaaccg gtgctggcga taagctgacc gtgttgtctc agttggggat
2761 cacgaccaac cagacgacgg gcgccctgga ttttgacagt acaaagttcg caactgccgt
2821 gaacgaaaaa cagttgggtg gagaaatcca gaccctgttc acaggcgaga atggcctgat
2881 cgagcgcatg cagaatgcac tcaatcccca cacggagacc agcaagaagg actccaatgg
2941 gaagaccgtc gataacatcc tgacggcgcg ctccaagaat cttgaaattc tcaaggcgaa
3001 actgagcgag gaccagttgg ccctggaccg ccgcatcgaa accctcaccg ccgtactgac

3061 caaaaagtac aacgacatgg acaccctggt aggcaggctg aaagccaccg ccagtaacat
3121 cacctccatg ttcgaagcga tgacggcaca gcagaaaaac agctgatttt cgaccgatgc
3181 aaaaagcccg gcaacgtttt gacaacgttc cgggctttcg gcttttcgac ctaaagtttt
3241 ttgacgcagc gtcgatacgc tgtttatacg aaccgaagtt ttttgatgag gtagaacatg
3301 aatccgatgt tagcccctcg ccaataccag aagattggcg cccaggcgca aacctccgaa
3361 gcaagtcccc atcgtctggt gcagatgctc atggaaggcg ggctggatcg cattgcccag
3421 gccaagggtg cgatggagcg caaggatatc gccaacaaag gcgtactgat cagcaaggcc
3481 atcggcatta tcggcggttt gcgtgaaggc ctggacctgg aaaaccaggc cgagtcggta
3541 accgagctgg ataacctcta cacctacatg atgaaacgcc tggccgaggc caacgccaag
3601 accgatccga agatcctcga cgaagtcgcc gatctgcttg gcacggtcaa ggaaggttgg
3661 gatgccatcg ctgtaccggg tccgcaattc taaggagtac gctcatgagt cttgtcttgc
3721 agcgaatcga acaaacccgt gatgccttgg tcgatgccct ggctgagcgt aactgggagg
3781 ccatcggtca gttggacctg gcctgccgtt cctgcatgga agacgtcctg agcgagtctc
3841 aggtggatga ggcggcgttg cggatcaatc ttgaggaatt gctgggggtg tatcggcaat
3901 tgctggaggc agcgactgga gagcgtcagg cgatcgtcga cgagatgcag cagatccatc
3961 aagcacagaa cgctgcaaag gtttaccatc tgttcggtta atgttcagtt aatccgagcc
4021 cgttgcgcca taaatttgac tgtgcacggt tttttgactt aactagtagc tgtttacaga
4081 tttaaggcgt ctacaggcat gacaagtctg caagcgtcta gcttgccccc taatttcggg
4141 cattgggttg actagggaag ttgctattgc atgtggcgtg aaaccaaaat tctcctgatc
4201 gatgacgata gcgtccgccg ccgcgacttg gcggtgatct taaattttct tggcgaagaa
4261 aatttaccct gcggaagcca tgactggcag caggccgtcg gctctttgtc gtccagtcgc
4321 gaggtcattt gtgtccttat cgggaccgtg aatgcccccg caacgctttt gggcttgcta
4381 aagacactct caacctggga tgagttcctt ccggttttgt taatgggcga aaattcttcc
4441 cttgacttgc ctgaggacca gcgccgccgg gtactttcca cgctcgaaat gccgcccagc
4501 tacagcaagc tgcttgattc gctgcaccgc gcgcaggttt atcgcgagat gtacgatcag
4561 gctcgcgagc gcggccggca tcgcgaaccc aacctgttcc gcagcctcgt cggcaccagc
4621 cgggcgatcc agcacgtgcg gcagatgatg cagcaggtcg ccgataccga cgccagcgtg
4681 ctgatcctgg gtgagtcggg caccggcaag gaagtggttg cgcgcaacct gcattaccac
4741 tccaagcgcc gcgaagcgcc gttcgtgcca gtcaactgtg gcgcgatccc ggccgagttg
4801 ctggagagcg agttgtttgg ccatgagaag ggcgccttta ccggcgcgat caccagtcgc
4861 gccgggcgtt tcgaactggc caatggcggt acgctgttcc tcgacgaaat cggtgacatg
4921 ccgctgccga tgcaggtcaa gctgttgcgg gtcctgcagg agcgcacctt cgagcgcgtg
4981 ggcagcaaca agacccagag cgtcgatgtg cgcatcatcg ccgccacgca caagaacctc
5041 gagagcatga tcgaggttgg cagcttccgc gaagacctct attaccgcct caacgttttc
5101 ccgatcgaaa tggcgccact gcgtgagcgc gtcgaagaca tcccgttgct gatgaacgag
5161 ctgatctcgc gcatggagca cgaaaagcgc ggttcgatcc gcttcaattc ggcggcgatc
5221 atgtccctgt gccgtcatgg ctggccgggc aacgtccggg aactggccaa cctggtggag
5281 cgcatggcga tcatgcaccc gtacggggtg atcggcgtga acgaactgcc gaagaaattc
5341 cgctacgtcg acgacgaaga cgagcaaatg gtcgacagcc tgcgcagcga tctggaagag
5401 cgggtggcaa tcaacggcca cacaccggat ttcaccgcca atgcgttgtt gccgcccgaa
5461 ggcctggacc tcaaggatta cctgggaggc ctggagcaag ggctgatcca gcaggcgctg
5521 gacgatgcca acggtatcgt tgcccgcgcg gcagaacgcc tgcgcatccg ccgtacctcc
5581 ctggtggagg agatgcgcaa gtacggcatg agccgccgtg aaggagatga acaggcggat
5641 gattgacgcc tgttttccaa ccgcctgatt tccaggcggt ttttttcgg cacgggtatt

5701 gctatagccc tcgcaacgtt ccgtttaact gacggtcagc caagcgagag agcacgatga
5761 ccctagccgc ccagatgtct cctgtccccg agccggaaca catgccgtcc gccgagcagg
5821 caagccggct tggacttgag caagcatttt cgctgttcaa ccagatgtcc agtcaactga
5881 cagactccta cagcctgctt gaagcccggg ttaccgagct caagggtgag ctggccgtgg
5941 tcagcgccca gcgcatgcag gagcttgcgg aaaaggaacg cctggctaat cgtctgcaaa
6001 atctcctcga cctgttgcct ggtggcgtta tcgtcatcga cgcccacggc cgtgtgcgcg
6061 aagccaaccc ggcggcttgc gagttgctcg gcctgccgtt ggaaggtgag ctgtggcggc
6121 atgtcatcgc ccgctgcttt gcgccccgtg aagacgacgg ccatgaagtg tccctcaagg
6181 acggtcggcg cctgtccatc tcgacacgtt cgctggatgc cgagccgggg cagttggtgc
6241 tgctcaatga cttgactgaa acccgtcacc tgcaagacca gttggctcgc catgagcgcc
6301 tgtcttccct ggggcggatg gtggcgtcgc tggcccatca gattcgtacg ccgctttctg
6361 ccgcgctgct ctatgccagt catctggctg aacagcaatt gccgatggac acccagcaac
6421 ggttcgccgg acgcttgaaa gagcgcctgc atgagctgga gcaccaggtg cgcgacatgc
6481 tggtattcaa tc

Secuencia gen *sss*

```
    ---------|---------|---------|---------|---------|---------|
  1 atggaacggcaactggacgcctactgcgaacacctgcgcagtgagcgccaggtgtcgccc 60
  1 M  E  R  Q  L  D  A  Y  C  E  H  L  R  S  E  R  Q  V  S  P   20

    ---------|---------|---------|---------|---------|---------|
 61 cacacgttgtcggcctatcgccgcgacctggaaaaagtgctgggctggtgccaaaagcaa 120
 21 H  T  L  S  A  Y  R  R  D  L  E  K  V  L  G  W  C  Q  K  Q   40

    ---------|---------|---------|---------|---------|---------|
121 aacatcggcagttgggcggccctggacatccagcgcttgcgcagcttgatcgcccgcctg 180
 41 N  I  G  S  W  A  A  L  D  I  Q  R  L  R  S  L  I  A  R  L   60

    ---------|---------|---------|---------|---------|---------|
181 catcaacaggggcaatcctcccgcagcctggcgcgcctgctgtcggcagtacgcggcctg 240
 61 H  Q  Q  G  Q  S  S  R  S  L  A  R  L  L  S  A  V  R  G  L   80

    ---------|---------|---------|---------|---------|---------|
241 tatcactacctcaatcgcgaaggcctgtgcgatcacgacccggccaccggcctggcgccg 300
 81 Y  H  Y  L  N  R  E  G  L  C  D  H  D  P  A  T  G  L  A  P   100

    ---------|---------|---------|---------|---------|---------|
301 cccaagggcgaacgccggttgccgaagaccctcgacaccgaccgcgccctgcaattgctt 360
101 P  K  G  E  R  R  L  P  K  T  L  D  T  D  R  A  L  Q  L  L   120

    ---------|---------|---------|---------|---------|---------|
361 gaaggtgccgtcgaggatgacttcctggcacagcgcgaccaggccattctcgagctgttc 420
121 E  G  A  V  E  D  D  F  L  A  Q  R  D  Q  A  I  L  E  L  F   140

    ---------|---------|---------|---------|---------|---------|
421 tattcttccggcctgcggctttcagagctgacggggcttaatctggatcaactggacctg 480
141 Y  S  S  G  L  R  L  S  E  L  T  G  L  N  L  D  Q  L  D  L   160

    ---------|---------|---------|---------|---------|---------|
481 gccgatggcatggtccaggtgctcggcaagggcagcaagacccgcctgttacccgttggc 540
161 A  D  G  M  V  Q  V  L  G  K  G  S  K  T  R  L  L  P  V  G   180

    ---------|---------|---------|---------|---------|---------|
541 cgcaaggcccgtgaagccctggagcaatggctggcgctgcgggcgctgaccaatcccgcc 600
181 R  K  A  R  E  A  L  E  Q  W  L  A  L  R  A  L  T  N  P  A   200

    ---------|---------|---------|---------|---------|---------|
601 gacgacgcggtcttcgtcagccaacaggggcgacgtctcggcccgcgggcgattcagctg 660
201 D  D  A  V  F  V  S  Q  Q  G  R  R  L  G  P  R  A  I  Q  L   220

    ---------|---------|---------|---------|---------|---------|
661 cgggtcaaggctgccggcgaacgggagctgggacagaacctgcacccgcacatgctcagg 720
221 R  V  K  A  A  G  E  R  E  L  G  Q  N  L  H  P  H  M  L  R   240

    ---------|---------|---------|---------|---------|---------|
721 cactccttcgccagccatctgcttgagtcctcccaggacctgcgcgccgttcaagagctg 780
241 H  S  F  A  S  H  L  L  E  S  S  Q  D  L  R  A  V  Q  E  L   260

    ---------|---------|---------|---------|---------|---------|
781 ctggggcactcggacatcaagaccacacagatctacacccacctggatttccagcacctg 840
261 L  G  H  S  D  I  K  T  T  Q  I  Y  T  H  L  D  F  Q  H  L   280

    ---------|---------|---------|---------|---------|---------|
841 gcaacggtctatgacagtgcccaccccagggccaaacgcatcaagggcgacgaatcatga 900
281 A  T  V  Y  D  S  A  H  P  R  A  K  R  I  K  G  D  E  S  *   299
```

Secuencia gen *xerD*

```
    ---------|---------|---------|---------|---------|---------|
  1 atgcctgccatcgaccatccgttgatagaccagtttctcgacgccctgtggctggagaag 60
  1 M  P  A  I  D  H  P  L  I  D  Q  F  L  D  A  L  W  L  E  K   20

    ---------|---------|---------|---------|---------|---------|
 61 ggcctgtccgataacacccgcgatgcctatcgcagcgacctggcgctgttcaacggttgg 120
 21 G  L  S  D  N  T  R  D  A  Y  R  S  D  L  A  L  F  N  G  W   40

    ---------|---------|---------|---------|---------|---------|
121 ttgcaggaaaatcacctggaactgatcaatgctggccgggaattgatcctcgatcacttg 180
 41 L  Q  E  N  H  L  E  L  I  N  A  G  R  E  L  I  L  D  H  L   60

    ---------|---------|---------|---------|---------|---------|
181 gcgtggcgcctggagcagaactacaagccgcgctcgaccgcgcgttttctctccggcctg 240
 61 A  W  R  L  E  Q  N  Y  K  P  R  S  T  A  R  F  L  S  G  L   80

    ---------|---------|---------|---------|---------|---------|
241 cgtgggttctatcgctatttgctgcgggaaaagctgattgcggtggacccgaccttgcgc 300
 81 R  G  F  Y  R  Y  L  L  R  E  K  L  I  A  V  D  P  T  L  R   100

    ---------|---------|---------|---------|---------|---------|
301 gtggaaatgccgcaactggggcgcccattgcccaagtccctgtcggaagccgatgtggag 360
101 V  E  M  P  Q  L  G  R  P  L  P  K  S  L  S  E  A  D  V  E   120

    ---------|---------|---------|---------|---------|---------|
361 gcgctgctggcggcacccgaccttagcgaagccatcggtcagcgcgaccgggccatgctg 420
121 A  L  L  A  A  P  D  L  S  E  A  I  G  Q  R  D  R  A  M  L   140

    ---------|---------|---------|---------|---------|---------|
421 gaagtcttgtacgcctgcggcctgcgggtcaccgagttgatcagcttgaccctggagcag 480
141 E  V  L  Y  A  C  G  L  R  V  T  E  L  I  S  L  T  L  E  Q   160

    ---------|---------|---------|---------|---------|---------|
481 gtcaacctgcgccagggcgtgctgcgggtgatgggtaaaggcagcaaggagcgcctggtg 540
161 V  N  L  R  Q  G  V  L  R  V  M  G  K  G  S  K  E  R  L  V   180

    ---------|---------|---------|---------|---------|---------|
541 ccgatgggggaggaagcgatcgtctgggtcgagcgctacgtgcgcgatgcccgccacgaa 600
181 P  M  G  E  E  A  I  V  W  V  E  R  Y  V  R  D  A  R  H  E   200

    ---------|---------|---------|---------|---------|---------|
601 ctgttgggggggcgccccagcgatgcgctgttccccagcctgcgcggcgaacagatgacc 660
201 L  L  G  G  R  P  S  D  A  L  F  P  S  L  R  G  E  Q  M  T   220

    ---------|---------|---------|---------|---------|---------|
661 cgccagaccttctggcaccggatcaagcaccaggccaaggtggccgggatcaacaaatcc 720
221 R  Q  T  F  W  H  R  I  K  H  Q  A  K  V  A  G  I  N  K  S   240

    ---------|---------|---------|---------|---------|---------|
721 ctctcgccccacaccttgcgccatgcctttgccacgcacctgctcaaccacggcgccgac 780
241 L  S  P  H  T  L  R  H  A  F  A  T  H  L  L  N  H  G  A  D   260

    ---------|---------|---------|---------|---------|---------|
781 ctgcgggtggtgcagatgctgctcggccacagcgacctgtccaccacccagatctacacc 840
261 L  R  V  V  Q  M  L  L  G  H  S  D  L  S  T  T  Q  I  Y  T   280

    ---------|---------|---------|---------|---------|-------
841 cacgtcgcccgggcgcggctgcaggacttgcatgccaaacaccatccgcgcggctga 897
281 H  V  A  R  A  R  L  Q  D  L  H  A  K  H  H  P  R  G  *   298
```

Secuencia gen *rsmA*

```
   ---------|---------|---------|---------|---------|---------|
  1 atggtgattctgactcgtcggtgtgcagaaagcctgattattggcgatggcgaaatcacc 60
  1 M  V  I  L  T  R  R  C  A  E  S  L  I  I  G  D  G  E  I  T   20

   ---------|---------|---------|---------|---------|---------|
 61 gtgaccgtgctcggcgtcaaaggaaaccaagtgcgtatcggcgtcaacgccccgaaagag 120
 21 V  T  V  L  G  V  K  G  N  Q  V  R  I  G  V  N  A  P  K  E   40

   ---------|---------|---------|---------|---------|---------|
121 gttgctgtacaccgcgaggaaatttacctgcgtatcaagaaagagaaggacgaagaacca 180
 41 V  A  V  H  R  E  E  I  Y  L  R  I  K  K  E  K  D  E  E  P   60

   ---------
181 agccattaa 189
 61 S  H  *   62
```

Secuencia gen *rsmE*

```
   ---------|---------|---------|---------|---------|---------|
  1 atgctgatactcacccgcaaagtcggtgaaagcataaacattggtgacgacatcacgatc 60
  1 M  L  I  L  T  R  K  V  G  E  S  I  N  I  G  D  D  I  T  I   20

   ---------|---------|---------|---------|---------|---------|
 61 accattctgggcgtaagcggccaacaggtccggatcggcatcaacgccccgaaaaacgtt 120
 21 T  I  L  G  V  S  G  Q  Q  V  R  I  G  I  N  A  P  K  N  V   40

   ---------|---------|---------|---------|---------|---------|
121 gcggtgcatcgcgaagagatttaccagcgtattcaggcgggcctcactgcccccgacaag 180
 41 A  V  H  R  E  E  I  Y  Q  R  I  Q  A  G  L  T  A  P  D  K   60

   ---------|-----
181 ccacaaacgccctga 195
 61 P  Q  T  P  *   64
```

Secuencia parcial gen *vfr*

```
    ---------|---------|---------|---------|---------|---------|
  1 atggttggtattacccccacgttcaagatcaagaatctcgacaaactcttgatgcattgc 60
  1 M  V  G  I  T  P  T  F  K  I  K  N  L  D  K  L  L  M  H  C   20

    ---------|---------|---------|---------|---------|---------|
 61 cagcgccgccgccatccagccaaacacaacatcatttgtgcaggagatcgttccgacacg 120
 21 Q  R  R  R  H  P  A  K  H  N  I  I  C  A  G  D  R  S  D  T   40

    ---------|---------|---------|---------|---------|---------|
121 ctgtttttcatcatcaagggttcggtcactatcctgatagaagatgacgacggtcgggaa 180
 41 L  F  F  I  I  K  G  S  V  T  I  L  I  E  D  D  D  G  R  E   60

    ---------|---------|---------|---------|---------|---------|
181 atgatcatcgcctacctcaattccggagatttttcggcgagctggggttgttcgaacag 240
 61 M  I  I  A  Y  L  N  S  G  D  F  F  G  E  L  G  L  F  E  Q   80

    ---------|---------|---------|---------|---------|---------|
241 gccggcaaggaacaggaacgcagtgcctgggtacggaccaagattgaatgtgaagtagcg 300
 81 A  G  K  E  Q  E  R  S  A  W  V  R  T  K  I  E  C  E  V  A   100

    ---------|---------|---------|---------|---------|---------|
301 gaaatcagctacgccaaattccgagaactgtccctgcaagacccggacattctttacgtc 360
101 E  I  S  Y  A  K  F  R  E  L  S  L  Q  D  P  D  I  L  Y  V   120

    ---------|---------|---------|---------|---------|---------|
361 ctcagcggacaaatcgcacagcgcctgcgcaataccacccgcaaggtcggcgatctcgca 420
121 L  S  G  Q  I  A  Q  R  L  R  N  T  T  R  K  V  G  D  L  A   140

    ---------|---------|---------|---------|---------|---------|
421 tttttcgacgtcaccggacgtgtcgcacgctgcctgttggaactgtgcaagcaacccgat 480
141 F  F  D  V  T  G  R  V  A  R  C  L  L  E  L  C  K  Q  P  D   160

    ---
481 gcc 483
161 A   161
```

Secuencia parcial gen *gacS*

```
    ---------|---------|---------|---------|---------|---------|
  1 gcggacgtgcgcgccgtaaccttcctcgctcccgaccgcctaccgctggcccacgccggc 60
  1 A  D  V  R  A  V  T  F  L  A  P  D  R  L  P  L  A  H  A  G   20

    ---------|---------|---------|---------|---------|---------|
 61 ccgaccatgctcaaccgggcgccggagggcaacagcacgcaactactgcaacgcaccggc 120
 21 P  T  M  L  N  R  A  P  E  G  N  S  T  Q  L  L  Q  R  T  G   40

    ---------|---------|---------|---------|---------|---------|
121 aacgatgccacgcgctacctgctgccggtattcggcaagcaccgcaacctggcgggcgaa 180
 41 N  D  A  T  R  Y  L  L  P  V  F  G  K  H  R  N  L  A  G  E   60

    ---------|---------|---------|---------|---------|---------|
181 ctgattcccgaagaagccgaccgcctgttgggctgggtcgagctggagttgtcccacagc 240
 61 L  I  P  E  E  A  D  R  L  L  G  W  V  E  L  E  L  S  H  S   80

    ---------|---------|---------|---------|---------|---------|
241 ggcatgttgctgcgcggttatcggagcttgttcgccagcctgctgttgatcggcgcgggc 300
 81 G  M  L  L  R  G  Y  R  S  L  F  A  S  L  L  L  I  G  A  G   100

    ---------|---------|---------|---------|---------|---------|
301 ctgtgcctgaccgcgctgttggccttgcgcatgggccgcaccatcaaccgtccgctgagc 360
101 L  C  L  T  A  L  L  A  L  R  M  G  R  T  I  N  R  P  L  S   120

    ---------|---------|---------|---------|---------|---------|
361 cagatcaagcaagccgtggcgcaactcaaggacggtcacctggaaacccgcctgccgccg 420
121 Q  I  K  Q  A  V  A  Q  L  K  D  G  H  L  E  T  R  L  P  P   140

    ---------|---------|---------|---------|---------|---------|
421 ctgggcagccaggagctggacgaactggcctcgggcatcaaccgcatggccagtaccttg 480
141 L  G  S  Q  E  L  D  E  L  A  S  G  I  N  R  M  A  S  T  L   160

    ---------|---------|---------|---------|---------|---------|
481 cagaacgcccaggaagaattgcagcacagcatcgaccaggccaccgaagacgtgcgccag 540
161 Q  N  A  Q  E  E  L  Q  H  S  I  D  Q  A  T  E  D  V  R  Q   180

    ---------|---------|---------|---------|---------|---------|
541 aatctggaaaccatcgagatccagaacatcgagctggacctggcccgcaaggaagccctg 600
181 N  L  E  T  I  E  I  Q  N  I  E  L  D  L  A  R  K  E  A  L   200

    ---------|---------|---------|---------|---------|---------|
601 gaagcgagccggatcaaatccgaatttctggcgaacatgagccatgaaatccgcacgccg 660
201 E  A  S  R  I  K  S  E  F  L  A  N  M  S  H  E  I  R  T  P   220

    ---------|---------|---------|---------|---------|---------|
661 ctcaacggtatcctcggttttacccacctgttgcaggaaagcgaactgaccccgcgccag 720
221 L  N  G  I  L  G  F  T  H  L  L  Q  E  S  E  L  T  P  R  Q   240

    ---------|---------|---------|---------|---------|---------|
721 ctcgactacctgggcaccatcgaaaaatccgccgacagcctgctggggatcatcaacgag 780
241 L  D  Y  L  G  T  I  E  K  S  A  D  S  L  L  G  I  I  N  E   260

    ---------|---------|---------|---------|---------|---------|
781 atcctcgacttttcgaagatcgaggccggcaagctggtgctcgacagcattccgttcaac 840
261 I  L  D  F  S  K  I  E  A  G  K  L  V  L  D  S  I  P  F  N   280

    ---------|---------|---------|---------|---------|---------|
841 ctgcgcgacctgctgcaggacaccctgaccatcctcgcccccgccgcccacgccaagcag 900
281 L  R  D  L  L  Q  D  T  L  T  I  L  A  P  A  A  H  A  K  Q   300
```

```
    ---------|---------|---------|---------|---------|---------|
 901 ctggaactggtgagcctggtctaccgggacacaccgttgtcgctggtgggcgatccgctg 960
 301 L  E  L  V  S  L  V  Y  R  D  T  P  L  S  L  V  G  D  P  L   320

    ---------|---------|---------|---------|---------|---------|
 961 cgcctcaagcagatcctcaccaacctggtgagcaacgccatcaagttcacccgcgagggc 1020
 321 R  L  K  Q  I  L  T  N  L  V  S  N  A  I  K  F  T  R  E  G   340

    ---------|---------|---------|---------|---------|---------|
1021 accatcgtcgcccgggccatgctcgaagacgagcacgaagacagcgtgcagttgcgcatc 1080
 341 T  I  V  A  R  A  M  L  E  D  E  H  E  D  S  V  Q  L  R  I   360

    ---------|---------|---------|---------|---------|---------|
1081 agcatccaggacacgggtatcggcctgtccaaccaggatgtgcgagcgctgttccaggcg 1140
 361 S  I  Q  D  T  G  I  G  L  S  N  Q  D  V  R  A  L  F  Q  A   380

    ---------|---------|---------|---------|---------|---------|
1141 ttcagccaggctgacaactcgctgtcgcggcaaccaggggggcaccgggttgggcctggtg 1200
 381 F  S  Q  A  D  N  S  L  S  R  Q  P  G  G  T  G  L  G  L  V   400

    ---------|---------|---------|---------|---------|---------|
1201 atttccaagcgcctggtggaacagatgggcggtgagatcggcgtcgacagcacaccgggc 1260
 401 I  S  K  R  L  V  E  Q  M  G  G  E  I  G  V  D  S  T  P  G   420

    ---------|---------|---------|---------|---------|---------|
1261 gagggttcggagttctggatcagcctgcgcctgcccaagacccgcgacgatgccgaggac 1320
 421 E  G  S  E  F  W  I  S  L  R  L  P  K  T  R  D  D  A  E  D   440

    ---------|---------|---------|---------|---------|---------|
1321 ctgcctggcccgccgttgctgggccggcgcgtggcggtgctggagaaccatgaactggcc 1380
 441 L  P  G  P  P  L  L  G  R  R  V  A  V  L  E  N  H  E  L  A   460

    ---------|---------|---------|---------|---------|---------|
1381 cgccaggccttgcagcaccagctcgaagactgcggcctgcaagtgacgccgttcaacacc 1440
 461 R  Q  A  L  Q  H  Q  L  E  D  C  G  L  Q  V  T  P  F  N  T   480

    ---------|---------|---------|---------|---------|---------|
1441 ctggagagcctgaccaacggcataaccatcgcccaccagaccgatcaggccatcgatctg 1500
 481 L  E  S  L  T  N  G  I  T  I  A  H  Q  T  D  Q  A  I  D  L   500

    ---------|---------|---------|---------|---------|---------|
1501 gccgtgttgggcatcaccagcaacgacatgccgccggagcgcctcaaccaacacatctgg 1560
 501 A  V  L  G  I  T  S  N  D  M  P  P  E  R  L  N  Q  H  I  W   520

    ---------|---------|---------|---------|---------|---------|
1561 gaccttgagcacctgggctgcaaagtactcgtgctgtgccccaccaccgaacagaccctg 1620
 521 D  L  E  H  L  G  C  K  V  L  V  L  C  P  T  T  E  Q  T  L   540

    ---------|---------|---------|---------|---------|---------|
1621 taccacctctcggtgcccaaccctcacagtcagttgcaggccaagccggcctgcacccgc 1680
 541 Y  H  L  S  V  P  N  P  H  S  Q  L  Q  A  K  P  A  C  T  R   560

    ---------|---------|---------|---------|---------|---------|
1681 aagttgcgccgctcgctgtcggacctggtcaacccacgcccgacccgcagcgaaccccac 1740
 561 K  L  R  R  S  L  S  D  L  V  N  P  R  P  T  R  S  E  P  H   580

    ---------|---------|------
1741 gagccgatttccagccgcgccccgaa 1766
 581 E  P  I  S  S  R  A  P    588
```